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RECOMBINANT PROTEIN CONTAINING A C-TERMINAL FRAGMENT OF *PLASMODIUM* MSP-1

The invention relates to novel active principles for vaccines derived from the major surface protein in merozoite forms of a *Plasmodium* which is infectious for mammals, especially man, more generally termed MSP-1

MSP-1 has already been the subject of a number of studies. It is synthesised in the schizont stage of *Plasmodium* type parasites, in particular *Plasmodium falciparum*, and is expressed in the form of one of the major surface constituents of merozoites both in the hepatic stage and in the erythrocytic stage of malaria (1, 2, 3, 4). Because of the protein's predominant character and conservation in all known *Plasmodium* species, it has been suggested that it could be a candidate for constituting anti-malarial vaccines (5, 6).

The same is true for fragments of that protein, particularly the natural cleavage products which are observed to form, for example during invasion by the parasite into erythrocytes of the infected host. Among such cleavage products are the C-terminal fragment with a molecular weight of 42 kDa (7, 8) which is itself cleaved once more into an N-terminal fragment with a conventional apparent molecular weight of 33 kDa and into a C-terminal fragment with a conventional apparent molecular weight of 19 kDa (9) which remains normally fixed to the parasite membrane after the modifications carried out on it, via glycosylphosphatidylinositol (GPI) groups (10, 11).

It is also found at the early ring stage of the intraerythrocytic development cycle (15, 16), where the observations were made that the 19 kDa fragment could play a role which is not yet known, but which is doubtless essential in re-invasive processes. This formed the basis for hypotheses formed in the past that that protein could constitute a particularly effective target for possible vaccines.

It should be understood that the references frequently made below to the p42 and p19 proteins from a certain type of *Plasmodium* are understood to refer to the corresponding C-terminal cleavage products of the MSP-1 protein of that *Plasmodium* or, by extension, to products containing substantially the same amino acid sequences, obtained by genetic recombination or by chemical synthesis using

conventional techniques, for example using the "Applied System" synthesiser, or by "Merrifield" type solid phase synthesis. For convenience, references to "recombinant p42" and "recombinant p19" refer to "p42" and "p19" obtained by techniques comprising at least one genetic engineering step

Faced with the difficulty of obtaining large quantities of parasites for *P. falciparum* and the impossibility of cultivating *P. vivax in vitro*, it has become clear that the only means of producing an anti-malaria vaccine is to resort to techniques which use recombinant proteins or peptides. However, MSP-1 is very difficult to produce whole because of it large size of about 200 kDa, a fact which has led researchers to study the C-terminal portion, the (still unknown) function of which is probably the more important.

Recombinant proteins concerning the C-terminal portion of the *P. falciparum* MSP-1 which have been produced and tested in the monkey (12, 40, 41) are

- a p19 fused with a glutathione-S-transferase produced in *E. coli* (40);
- a p42 fused with a glutathione-S-transferase produced in *E. coli*,
- a p19 fused with a polypeptide from a tetanic anatoxin and carrying auxiliary T cell epitopes produced in *S. cerevisiae* (12);
- a p42 produce in a baculovirus system (41).

A composition containing a p19 protein fused with a glutathione-S-transferase produced in *E. coli* combined with alum or liposomes did not exhibit a protective effect in any of six vaccinated *Aotus nancymai* monkeys (40).

A composition containing a p42 protein fused with a glutathione-S-transferase produced in *E. coli* combined with a Freund complete adjuvant did not exhibit a protective effect in two types of *Aotus* monkeys (*A. nancymai* and *A. vociferans*) when administered to them. The p19 protein produced in *S. cerevisiae* exhibited a protective effect in two *A. nancymai* type *Aotus* monkeys (12). In contrast, there was no protective effect in two *A. vociferans* type *Aotus* monkeys.

Some researchers (Chang et al.) have also reported immunisation tests carried out in the rabbit using a recombinant p42 protein produced in a baculovirus system and containing one amino acid sequence in common with *P. falciparum*

(18). Thus these latter authors indicate that in the rabbit that recombinant p42 behaves substantially in the same way as the entire recombinant MSP-1 protein (gp195). This p42 protein in combination with a Freund complete adjuvant has been the subject matter of a vaccination test in a non-human primate susceptible to infection by *P. falciparum*. *Aotus. lemurinus grisemembra* (40). The results showed that 2 of 3 animals were completely protected and the third, while exhibiting a parasitemia which resembled that of the controls, had a longer latent period. It is nevertheless dangerous to conclude a protective nature in man for the antibodies thus induced to counter the parasites themselves. It should be remembered that there are currently no very satisfactory experimental models in the primate for *P. vivax* and *P. falciparum*. The *Saimiri* model, developed for *P. falciparum* and *P. vivax*, and the *Aotus* model for *P. falciparum*, are artificial systems requiring the parasite strains to be adapted and often requiring splenectomy of the animals to obtain significant parasitemia. As a result, the vaccination results from such models can only have a limited predictive value for man.

In addition to questioning what the real vaccination rate which could possibly be obtained with such recombinant proteins would be, the presence in p42s from *Plasmodiums* of the same species, and more particularly in the corresponding p33s, of hypervariable regions could in many cases render uncertain the immunoprotective efficacy of antibodies induced in individuals vaccinated with a p42 from a *Plasmodium* strain against an infection by other strains of the same species (13).

The high polymorphism of central regions of the p42 could even play a significant role in immune escape, often observed for that type of parasite.

However, it appears that p42s from a variety of *Plasmodiums* which are infectious for man comprise hypervariable regions principally concentrated in their III regions, and more still in their respective II regions: see the publication by S. Longacre (13) in which the p42 sequences from *P. cynomolgi*, *P. vivax* (Belem) and *P. vivax* (Sal-I) were aligned. The "consensus" sequence of the accompanying Figure 4 added to the p42 sequences of those three parasites bears witness to this.

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The article by S. Longacre (13) describes the conditions under which the regions were determined. It should be noted that accompanying Figure 4 is nothing less than a reproduction of Figure 1 in the Longacre article. The reader is invited to refer to the key to that Figure. This also highlights the relative sizes of the four regions of the p42 (region IV corresponding to the sequence of the p19) expressed as percentages with respect to the size of the sequence coding for the whole of the p42.

Figure 4 of the present application also shows that the percentage homology is high between the sequences of regions I of *P. cynomolgi* and *P. vivax*. 84% in region I. 86% in region IV. In contrast, this percentage homology decreases substantially in region III (69%) and more still in region II (47%).

A first aim of the invention is thus to provide active principles for vaccines from p42 which are more capable of protecting the host against immune escape as discussed above.

It follows that fragmentation of p42 as just envisaged can also be extended to *P. falciparum*, the parasite which is principally responsible for acute forms of malaria in man, all the more if the locations of the zones separating regions I. II. III and IV of the constituent sequences of *P. cynomogi* and the two varieties of *P. vivax* have been determined by analogy with corresponding sites already identified in *P. falciparum*, as described in (34) and (35).

More particularly, the invention provides vaccinating compositions against a *Plasmodium* type parasite which is infectious for man, containing as an active principle a recombinant protein which may or may not be glycosylated, whose essential constituent polypeptide sequence is,

- either that of the p42 from which region II and, if necessary, all or part of region III have been deleted;
- or that of a portion of that fragment which is also capable of inducing an immune response which can inhibit *in vivo* parasitemia due to the corresponding parasite;

- or that of an immunologically equivalent peptide of said p42 fragment or said portion of that fragment; and

said recombinant protein further comprises conformational epitopes which are unstable in a reducing medium and which preferably constitute the majority of the epitopes recognised by human antiserums formed against the corresponding *Plasmodium*

More particularly, then, the invention provides a recombinant protein, which may or may not be glycosylated, originating from p42 and containing both the essential portions of region I and region IV defined above to constitute immunogenic compositions, in particular vaccines.

If necessary, said recombinant protein, which may or may not be glycosylated, also contains the conserved portion of region III which is located on the C-terminal side of the p33, close to the p19, in particular that which extends between amino acids 255 to 273, or more particularly still between amino acids 255 to 270 (see the numbering of the consensus region in Figure 4).

In other words, all or part of the less conserved portion of region III can be deleted from the N-terminal portion of region III.

For convenience, reference will frequently be made below to a "partially deleted p42" to designate the modified p42, as defined above.

The presence in this active principle of said conformational epitopes could play an important role in the protective efficacy which it can confer on the vaccinated host. They are particularly found in the active principles which exhibit the other characteristics defined above, when they are produced in a baculovirus vector system. If needs be, it is mentioned below that the expression "baculovirus vector system" means the ensemble constituted by the baculovirus type vector itself and the cell lines, in particular cells of insects transfectable by a baculovirus modified by a sequence to be transferred to these cell lines resulting in expression of that transferred sequence. Preferred examples of these two partners in the baculovirus system have been described in the article by Longacre et al. (19). The same system was used in the examples below. It naturally follows, of course, that

variations in the baculovirus and in the cells which can be infected by the baculovirus can be made in place of those selected.

The unstable character of these conformational epitopes in a reducing medium can be demonstrated by the test described below in the examples, in particular in the presence of ß-mercaptoethanol.

From this viewpoint, recombinant proteins derived from the recombinant p42 produced by Longacre et al. (14) can be used in such compositions. It should be remembered that S. Longacre et al. succeeded in producing a recombinant p19 from the MSP-1 of *P. vivax* in a baculovirus vector system containing a nucleotide sequence coding for the p19 of *Plasmodium vivax*, in particular by transfecting cultures of insect cells [*Spodoptera frugiperda (Sf9)* line] with baculovirus vectors containing, under the control of the polyhedrin promoter, a sequence coding for the peptide fragments defined below, with the sequences being placed in the following order in the baculovirus vector used:

- a 35 base pair 5' terminal fragment of the polyhedrin signal sequence, in which the methionine codon for initiating expression of this protein had been mutated (to ATT);
- a 5'-terminal nucleotide fragment coding for a 32 amino acid peptide corresponding to the N-terminal portion of MSP-1, including the MSP-1 signal peptide;
- either a nucleotide sequence coding for p19, or a sequence coding for the p42 of the MSP-1 protein of *Plasmodium vivax*, these sequences also being provided, depending on the case, with ("anchored" forms) or deprived of (soluble forms) 3' end regions of these nucleotide sequences for which the end C-terminal expression products are reputed to play an essential role in anchoring the final p19 protein to the parasite membrane;
- 2 TAA stop codons.

For p42, the sequences derived from the C-terminal region of MSP-1 would extend as a result from amino acid Asp 1325 to amino acid Leu 1726 (anchored form) or to amino acid Ser 1705 (soluble form) and for p19, the

sequences would extend from amino acid Ile 1602 to amino acid Leu 1726 (anchored form) or to amino acid Ser 1705 (soluble form) it being understood that the complete amino acid sequences of p42 and p19 for which the initial and terminal amino acids have been indicated above follow from the gene of the Belem isolate of *P. vivax* which has been sequenced (20)

Similar results were obtained using, in the same vector systems, nucleotide sequences coding for the p19 and p42 of *Plasmodium cynomolgi*. The interest in *P. cynomolgi* is twofold: it is a parasitic species very close to *P. vivax* which is highly infectious for the macaque. It can also infect man. Further, access to the natural hosts of *P. cynomolgi*, rhesus monkeys and toque macaques, is also possible, to test the efficacy of the protection of MSP-1 from *P. cynomolgi* in natural systems. The rhesus monkey is considered to be one of the most representative species for immune reactions in man.

In particular, excellent results have been obtained in vaccination tests carried out using the toque macaque with two recombinant polypeptides, soluble p42 and p19 derived from *P. cynomolgi*, respectively produced in a baculovirus system and purified on an affinity column with monoclonal antibodies recognising the corresponding regions of the native MSP-1 protein. The following observations were made: six monkeys immunised with p19 alone (three monkeys) and p19 and p42 together (three monkeys) all exhibited practically sterile immunity after challenge infection. The results obtained in the three monkeys immunised with p42 were less significant. Two of them were as above, but since the third exhibited a lower parasitemia than the controls immunised with a PBS buffer in the presence of Freund adjuvant (3 monkeys) or not immunised (3 monkeys), it was less clear.

The particularly effective test results carried out with the macaque with recombinant polypeptides produced in a baculovirus system using a p42 in combination with a recombinant p19 from *P. cynomolgi* showed that recombinant polypeptides respectively containing recombinant p42s from other *Plasmodiums* must behave in the same manner. These tests are more meaningful for malaria in

man than the results from tests carried out with *P. vivax* or *P. falciparum* in their "artificial hosts".

Baculovirus recombinant proteins derived from a C-terminal MSP-1 portion (p42), more particularly partially deleted p42s, have a very significant antimalarial protective effect in a natural system, which constitutes the most representative model for evaluating the protective effect of MSP-1 for man.

The protective effect obtained can be further improved if the partially deleted p42 form is deprived of the hypervariable region of the N-terminal portion of p42, the effect of which can be deleterious in natural situations in which the vaccinated subject is confronted by a great deal of polymorphism.

However, deletion of region II and all or part of region III normally produces the best results. It is clear that the skilled person would have no difficulty in producing fusion proteins between regions I and IV of the corresponding p42s, or even between a region I and a region IV respectively originating from two p42s from two different varieties of *Plasmodium*. It goes without saying that these fusion proteins can also contain binding elements corresponding to portions of region II, preferably to region III, preferably selected from the best conserved. As an example, the C-terminal polypeptide sequence of p33, when it is present, contains less than 50 amino acid residues, or even less than 35, or even less than 10 amino acid residues.

The polypeptide sequence of the partially deleted p42 protein does not need to comprise all of the sequence coding for p19 (or region IV), naturally providing that the latter retains the ability to induce antibodies which protect against the parasite. In particular, the molecular weight of the "fragment portion" is 10 to 25 kDa, in particular 10 to 15 kDa. Preferably, this polypeptide fragment portion contains at least one of the two EGF (Epidermal Growth Factor) regions.

Naturally, the same observations apply to region I of the recombinant protein of the invention.

Clearly, the skilled person could distinguish between active fragments and those which ceased to be so, in particular experimentally by producing modified vectors containing, for example, inserts comprising portions ofp42, in particular of deleted p42,

of different lengths, respectively produced from the sequence coding for p42, if necessary partially deleted, by reaction with appropriate restriction enzymes, or by exonucleolytic enzymes which would be kept in contact with the fragment coding for the initial p42, if necessary partially deleted, for differing periods; the capacity of the expression products from these inserts in the corresponding eukaryotic cells, in particular in insect cells, transformed by the corresponding modified vectors, to exert a protective effect can then be tested, in particular under the experimental conditions which are described below in the examples. In particular, the expression products of these inserts must be able to inhibit a parasitemia induced *in vivo* by the corresponding whole parasite.

Thus, the invention includes all immunogenic or vaccinating compositions in which the essential constituent polypeptide sequence of the active principle is constituted by a peptide which can induce a cellular and/or humoral type immunological response equivalent to that produced by the partially deleted protein as defined above, provided that the addition, deletion or substitution in the sequence of certain amino acids by others would not cause a large modification of the capacity of the modified peptide - hereinafter termed the "immunologically equivalent peptide" - to inhibit said parasitemia.

The partially deleted p42 can naturally also be associated at the N-terminal side or the C-terminal side or via a peptide bond to a further plasmoidal protein fragment having a vaccinating potential (such as Duffy binding protein from *P. vivax* (29) or EBA-175 from *P. falciparum* (30) and (31), one region of which is specifically rich in cysteine), provided that its capacity to inhibit parasitemia normally introduced *in vivo* by the corresponding parasite is not altered but is amplified.

Upstream of its N-terminal end, the fragment coding for the partially deleted p42 or a portion thereof can also contain a peptide sequence which is different again, for example a C-terminal fragment of the signal peptide of the MSP-1 protein. This sequence preferably comprises less than 50 amino acids, for example 10 to 35 amino acids.

These observations pertain in similar fashion to the partially deleted p42s from other *Plasmodium*, in particular *P. falciparum*, the dominant species of the parasites, responsible for one of the most serious forms of malaria

However, the techniques summarised above for producing a recombinant p42 from *P. vivax* or *P. cynomolgi* in a baculovirus system are difficult to transpose unchanged to producing a recombinant p42 of *P. falciparum* in a satisfactory yield, if only to obtain appreciable quantities which will allow immunoprotective tests to be carried out

The invention also provides a process which overcomes this problem to a large extent. It also becomes possible to obtain much higher yields of *P. falciparum* p42 - and other *Plasmodiums* where similar difficulties are encountered - using a synthetic nucleotide sequence substituting for the natural nucleotide sequence coding for the p42 of *Plasmodium falciparum* in an expression vector of a baculovirus system, this synthetic nucleotide sequence coding for the same p42, but being characterized by a higher proportion of G and C nucleotides than in the natural nucleotide sequence.

It is clear to the skilled person that this result can be obtained by deriving benefit both from its ability to synthesise DNAs by nucleotide synthesis and by selecting from the possibilities offered by the genetic code to substitute, each time the genetic code allows it, synthetic codons having higher amounts of C and/or G than natural codons which, in native sequences coding for the corresponding native p42s, code for the same amino acid.

It follows that the same observations can be extended to p42s from which sequences have been partially deleted, as defined above

In other words, the invention follows from the discovery that expression of a nucleotide sequence coding for a partially deleted or non partially deleted p42 in a baculovirus system is apparently linked to an improved compatibility of successive codons in the nucleotide sequence to express using the "cellular machinery" of the host cells transformable by the baculovirus, in the manner of that observed for the natural nucleotide sequences normally contained in these baculovirus and expressed in the infected host cells: hence the poor expression, or even total absence of expression of a native *P. falciparum* nucleotide sequence; hence also a possible

explanation of the more effective expression observed by Longacre et al (19) for the p42 of *P. vivax* in a baculovirus system and, as the inventors have also shown, of the *P. cynomolgi* sequence from corresponding native p42 nucleotide sequences, because of their relatively much higher amounts of G and C nucleotides than those of the native nucleotide sequences coding for the p42 of *P. falciparum*

The invention thus more generally provides a recombinant baculovirus type modified vector containing, under the control of a promoter contained in said vector and able to be recognised by cells transfectable by said vector, a first nucleotide sequence coding for a signal peptide exploitable by a baculovirus system, characterized by a second nucleotide sequence downstream of the first, also under the control of said promoter and coding for a peptide sequence, but comprising, in its own constitutive sequence,

- either that of a peptide fragment coding for p42 or a p42 from which region II and, if necessary, all or part of region III have been deleted,
- or that of a portion of that peptide fragment provided that the expression product from the second sequence in a baculovirus system is also capable of inhibiting a parasitemia normally induced *in vivo* by the corresponding parasite;
- or that of an immunologically equivalent peptide derived from said C-terminal peptide fragment (p19) or said peptide fragment portion by addition, deletion or substitution of amino acids not resulting in a large modification of the capacity of said immunologically equivalent peptide to induce a cellular and/or humoral type immunological response similar to that produced by said p19 peptide fragment or said portion of said fragment; and

said nucleotide sequence having, if necessary, a G and C nucleotide content in the range 40% to 60%, preferably at least 50%, of the totality of the nucleotides from which it is constituted. This sequence can be obtained by constructing a synthetic gene in which the natural codons have been changed for codons which are rich in G/C without modifying their translation (maintaining the peptide sequence).

The nucleotide sequence, provided by a synthetic DNA, may have at least 10% of its codons modified with respect to the natural cDNA gene sequence while

retaining the characteristics of the natural translated sequence. i.e.. maintaining the amino acid sequence.

It is not excluded that this G and C nucleotide content could be further increased provided that the modifications resulting therefrom as to the amino acid sequence of the recombinant peptide - or immunologically equivalent peptide - produced did not result in a loss of immunological properties. or protective properties. for the recombinant proteins formed. in particular in the tests which will be described below.

These observations naturally apply to other *Plasmodium* which are infectious for man. in particular those where the native nucleotide sequences coding for the corresponding p42s. if necessary partially deleted. would have T and A nucleotide contents which were poorly compatible with effective expression in a baculovirus system.

The sequence coding for the signal used can be that normally associated with the native sequence of the *Plasmodium* concerned. But it can also originate from another *Plasmodium*. for example *P. vivax* or *P. cynomolgi* or another organism if it can be recognised as a signal in a baculovirus system.

The sequence coding for p42 or a portion thereof in the vector under consideration is. if necessary, deprived of the anchoring sequence of the native protein to the parasite from which it originates. in which case the expressed protein is gen rally excreted into the culture medium (soluble form).

The invention also concerns vectors in which the coding sequence contains the terminal 3'end sequence coding for the hydrophobic C'-terminal end sequence of the p19 which is normally implicated in the induction of anchoring the native protein to the cell membrane of the host in which it is expressed. This 3'-terminal end region can also be heterologous relative to the sequence coding for the remainder of the corresponding p42, for example corresponding to the 3'-terminal sequence from *P. vivax* or from another organism when it codes for a sequence which anchors the whole of the recombinant protein produced to the cell membrane of the host of the baculovirus system used. An example of such anchoring

sequences is the GPI of the CD59 antigen which can be expressed in the cells of *Spodoptera frugiperda* (32) type insects or the GPI of a CD14 human protein (33)

The invention also, naturally, concerns recombinant proteins, these proteins comprising conformational epitopes recognised by human serums formed against the corresponding *Plasmodium*.

In general, the invention also concerns any recombinant protein of the type indicated above, provided that it comprises conformational epitopes such as those produced in the baculovirus system, in particular those which are unstable in a reducing medium.

The invention also, naturally, concerns said recombinant proteins, whether they are in their soluble form or in the form provided with an anchoring region, in particular to cellular hosts used in the baculovirus system.

The invention also encompasses any product of conjugation between a p42 or a partially deleted p42 as defined above, and a carrier molecule - for example a polylysine-alanine - for use in the production of vaccines, by means of covalent or non covalent bonds. Vaccinating compositions using them also form part of the invention.

The invention still further concerns vaccine compositions using these recombinant or conjugated proteins, including proteins from *Plasmodium vivax*.

The invention also encompasses compositions in which the recombinant proteins defined above are associated with an adjuvant, for example an alum. Recombinant proteins containing the C-terminal end region allowing them to anchor to the membrane of the cells in which they are produced are advantageously used in combination with lipids which can form liposomes appropriate to the production of vaccines. Without being limiting, lipids described, for example, in the publication entitled "Les liposomes aspects technologique, biologique et pharmacologique" [Liposomes: technological, biological and pharmacological aspects] by J. Delattre et al., INSERM, 1993, can be used.

The presence f the anchoring region in the recombinant protein, whether it is a homologous or heterologous anchoring region as regards the vaccinating portion proper, encourages the production of cytophilic antibodies, in particular

IgG_{2a} and IgG_{2b} type in the mouse, which could have a particularly high protective activity, so that associating the active principles of the vaccines so constituted with adjuvants other than the lipids used to constitute the liposome forms could be dispensed with. This amounts to a major advantage, since liposomes can be lyophilised under conditions which enable them to be stored and transported, without the need for chains of cold storage means.

Other characteristics of the invention will become clear from the following description of examples of recombinant proteins using the recombinant proteins the active sequences of which either contain those of the p42, or are limited to those of the corresponding p19 proteins. While the subject matter of these examples is not strictly linked to the claims which follow, the examples nevertheless contribute to establishing the operational character of the invention of the present application

Description of the PfMSP1$_{p19}$S (soluble) construction (soluble p19 from *P. falciparum*)

The recombinant construction PfMSP1$_{p19}$S contains the DNA corresponding to 8 base pairs of the leader sequence and the first 32 amino acids of the MSP-1 of *Plasmodium vivax* from Met$_1$ to Asp$_{32}$ (Belem isolate, Del Portillo et al., 1991, P. N. A. S., 88, 4030) followed by GluPhe due to the EcoR1 site connecting the two fragments. This is followed by the synthetic gene described in Figure 1, coding the *Plasmodium falciparum* MSP1$_{p19}$ from Asn$_{1613}$ to Ser$_{1705}$ (Uganda-Palo Alto isolate; Chang et al., 1988, Exp. Parasitol., 67, 1). The construction is terminated by two TAA stop codons. This construction gave rise to a recombinant protein which was secreted in the culture supernatant from infected cells.

In the same manner and for comparison, a recombinant construction was produced under conditions which were similar to those used to produce the p19 above, but working with a coding sequence consisting of a direct copy of the corresponding DNA of the *P. falciparum* strain (FUP) described by Chang et al., Exp. Parasit. 67,1; 1989. The natural gene copy (from asparagine 1613 to serine 1705) was formed from the native gene by PCR.

Figure 1A shows the sequences of both the synthetic gene (Bac19) and the "native gene" (PF19).

It can be seen that 57 codons of the 93 codons of the native sequence coding for the p19 from *P. falciparum* were modified (the third nucleotide in 55 of them and the first and third nucleotides in the other 2 codons). New codons were added to the 5' end to introduce the peptide signal under the conditions indicated above and to introduce an EcoRI site for cloning, and similarly two stop codons were added which were not present in the *P. falciparum* p19 to obtain expression termination signals. The individual letters placed above successive codons correspond to the respective successive amino acids. Asterisks (*) show the stop codons. Vertical lines indicate the nucleotides which are the same in the two sequences

Description of the PfMSP1$_{p19}$A construction (anchored GPI) (anchored p19 of *P. falciparum*)

The PfMSP1$_{p19}$A construction had the characteristics of that above except that the synthetic sequence (**Figure 1B**) codes for the MSP1$_{p19}$ of *Plasmodium falciparum* (Uganda-Palo Alto isolate) from Asn$_{1613}$ to Ile$_{1726}$ followed by two TAA stop codons. This construction gave rise to a recombinant protein which was anchored in the plasma membrane of infected cells by a glycosyl phosphatidyl inositol (GPI) type structure.

Figure 1C represents the PfMSP1$_{p19}$S recombinant protein sequence before cutting out the signal sequence.

Figure 1D represents the PfMSP1$_{p19}$S recombinant protein sequence after cutting out the signal sequence.

The amino acids underlined in Figures 1C and 1D originate from the EcoRI site used to join the nucleotide sequences derived from the N-terminal portion of the MSP-1 of *P. vivax* (with signal sequence) and the MSP-1$_{p19}$ of *P. falciparum*.

Figure 2 - The soluble recombinant PfMSP1$_{p19}$ antige· purified by immunoaffinity was analysed by immunoblot using SDS-PAGE in the presence (reduced) or absence (non reduced) of B-mercaptoethanol. Samples were charged onto gel after heating to 95°C in the presence of 2% SDS. Under these conditions only covalent type bonds (disulphide bridges) can resist disaggregation. The left hand blot was reveal d with a monoclonal antibody which reacted with a linear epitope of natural p19. The right hand blot was r vealed with a mixture of 13

human antisera originating from subjects with acquired immunity to malaria due to *Plasmodium falciparum*. These results show that the recombinant baculovirus molecule can reproduce conformational epitopes in the form of a polymer the majority of which are recognised by human antiserum.

Figure 3 - The soluble $PvMSP1_{p42}$ recombinant antigen (Longacre et al., 1994, op. Cit.) was incubated for 5 hours at 37°C in the presence of protein fractions derived from merozoites of *P. falciparum* and separated by isolectrofocussing. The samples were then analysed by immunoblot in the presence (reduced) or absence (non reduced) of B-mercaptoethanol. Isolectrofocussing fractions 5 to 12, and two total merozoite extracts made in the presence (Tex) or absence (T) of detergent, were analysed. The immunoblot was revealed with monoclonal antibodies specific for $MSP1_{p42}$ and $_{p19}$ of *P. vivax*. The results suggest that there is a proteolytic activity in the *P. falciparum* merozoites which can be extracted with detergent. Digestion of p42 in certain fractions appears to cause polymerisation of the digestion products (p19); this polymerisation is probably linked to the formation of disulphide bridges since in the presence of B-mercaptoethanol, the high molecular weight forms disappear in favour of a molecule of about 19 kDa (Tex-R). The p19 polymerisation observed in these experiments could thus be an intrinsic property of this molecule *in vivo*.

Description of the $PcMSP1_{p19}S$ (soluble) construction (soluble p19 of *P. cynomolgi*)

The DNA used for the above construction was obtained from a clone of the *Plasmodium cynomolgi ceylonesis* strain (22-23). This strain had been maintained by successive passages through its natural host (*Macaca sinica*) and cyclic transmissions via mosquitoes (27).

Blood parasites in the mature schizont stage were obtained from infected monkeys when the parasitemia had attained a level of 5%. They were then purified using the methods described in (25). The DNA was then extracted as described in (26).

A 1200 base pair fragment was produced using a PCR reaction using the oligonucleotides underlined in **Figure 4** originating from *P. vivax*. The 5'

oligonucleotide comprised an EcoRI restriction site and the 3' oligonucleotide comprised two synthetic TAA stop codons followed by a BglII restriction site. This fragment was introduced by ligation and via these EcoRI and BglII sites into the $pVLSV_{200}$ plasmid already containing the signal sequence for the MSP-1 protein of *P. vivax* (19). The new plasmid ($pVLSV_{200}C_{42}$) was used to analyse the DNA sequences.

The *P cynomolgi* sequences and the corresponding *P. vivax* sequences were aligned. The black arrows designate the presumed primary and secondary cleavage sites. They were determined by analogy with known sites in *P. falciparum* (27, 28). The vertical lines and horizontal arrows localise the limits of the four regions which were studied. Region 4 corresponded to the sequence coding for the *P. cynomolgi* p19. Glycosylation sites are boxed and the conserved cysteines are underlined. The lower portion of **Figure 4** shows the percentage identity between the two isolates of *P. vivax* and *P. cynomolgi*.

The recombinant construction $PcMSP1_{p19}S$ contains the DNA corresponding to 8 base pairs of the leader sequence and the first 32 amino acids of the MSP-1 of *Plasmodium vivax* from Met_1 to Asp_{32} (Belem isolate: Del Portillo et al., 1991, P. N. A. S., 88, 4030) followed by GluPhe, due to the EcoR1 site, connecting the two fragments. This is followed by the sequence coding for the *Plasmodium cynomolgi* $MSP1_{p19}$ from Lys_{276} to Ser_{380} (Ceylon strain). The construction was terminated by two TAA stop codons. This construction gave rise to a recombinant protein which was secreted in the culture supernatant of infected cells.

Purification of recombinant PfMSP1p19 protein by immunoaffinity chromatography with a monoclonal antibody specifically recognising the p19 of *Plasmodium falciparum*

The chromatographic resin was prepared by binding 70 mg of a monoclonal antibody (obtained from a G17.12 hybridoma deposited at the CNCM [National Collection of Microorganism Cultures] (Paris, France) on the 14th February 1997, registration number I-1846; this G17.12 hybridoma was constructed from X63 Ag8 653 myeloma producing IgG 2a/k recognising the *P. falciparum* p19) to 3 g of activated CNBr-Sepharose 4B (Pharmacia) using standard methods detailed in the procedure employed by Pharmacia. The culture supernatants containing the soluble

PfMSP1p19 were batch incubated with the chromatographic resin for 16 hours at 4°C The column was washed once with 20 volumes of 0.05% NP40, 0.5 M of NaCl, PBS; once with 5 volumes of PBS and once with 2 volumes of 10 mM sodium phosphate, pH 6.8. Elution was carried out with 30 ml of 0.2 M glycine, pH 2.2 The eluate was neutralised with 1 M sodium phosphate, pH 7.7 then concentrated by ultrafiltration and dialysed against PBS. To purify the anchored PfMSP1p19, all of the washing and elution solutions contained a supplement of 0.1% of 3-(dimethyl-dodecylammonio)-propane sulphonate (Fluka).

Recombinant *Plasmodium vivax* (p42 and p19) MSP1 vaccination test in the squirrel monkey *Saimiri sciureus*

This vaccination test was carried out on male non splenectomised 2 to 3 year old *Saimiri sciureus boliviensis* monkeys. Three monkeys were injected 3 times intramuscularly at 3 week intervals with a mixture of about 50 to 100 μg each of recombinant soluble $PvMSP1_{p42}$ and $_{p19}$ (19), purified by immunoaffinity. Complete and incomplete Freund adjuvant was used as follows: 1^{st} injection: 1:1 FCA/FIA; 2^{nd} injection: 1:4 FCA/FIA; 3^{rd} injection: FIA. These adjuvant compositions were then mixed 1:1 with the antigen in PBS. Five control monkeys received the glutathione-S-transferase (GST) antigen produced in *E. coli* using the same protocol. The challenge infection was carried out by injecting 2×10^6 red blood cells infected with an adapted *Plasmodium vivax* strain (Belem) 2.5 weeks after the final injection. The protection was evaluated by determining parasitemia daily in all animals by examining smears stained with Giemsa.

The curves in **Figure 5** show the variation in the measured parasitemia as the number of parasited red blood cells per microlitre of blood (up the ordinate, logarithmic scale) as a function of the time passed after infection (in days). Curve A corresponds to the average values observed in the three vaccinated monkeys; curve B corresponds to the average values in the five control monkeys.

An examination of the Figure shows that the effect of the vaccination was to greatly reduce the parasitemia.

Recombinant *Plasmodium cynomolgi* (p42 and p19) MSP1 vaccination test in the toque macaque *Macaca sinica*

Fifteen captured monkeys were used as follows (1) 3 animals injected with 100 µg of soluble $PcMSP1_{p42}$; 3 animals injected with 35 µg (1st injection) or 50 µg (2nd and 3rd injections) of soluble $PcMSP1_{p42}$; (3) 3 animals injected with a mixture of $PcMSP1_{p42}$ and $_{p19}$; (4) 3 animals injected with adjuvant plus PBS. (5) 3 animals not injected Complete and incomplete Freund adjuvant was used in the protocol described above Injections were intramuscular at 4 week intervals The challenge infection was made by injecting 2×10^5 red blood cells infected with *Plasmodium cynomolgi* 4 weeks after the last injection. Protection was evaluated by determining parasitemia daily in all animals by examining the parasitemia with Giemsa. Parasitemia were classified as negative only after counting 400 smear fields The parasitemia were expressed as a percentage of parasitised red blood cells.

Figures 6A - 6G show the results obtained. Each of them shows parasitemia (expressed as the percentage of parasitised red blood cells up the ordinate on a logarithmic scale) observed in the challenge animals as a function of the time after infection (in days along the abscissa).

The results relate to:

- in **Figure 6A**; non vaccinated control animals;
- **Figure 6B** relates to animals which received a saline solution also containing Freund adjuvant;
- **Figure 6C** is a superposition of figures 6A and 6B. with the aim of highlighting the relative results resulting from administration of Freund adjuvant to the animals (the variations are clearly not significant);
- **Figure 6D** provides the results obtained at the end of vaccination with p42;
- **Figure 6E** concerns animals vaccinated with p19 alone;
- finally, **Figure 6F** concerns animals vaccinated with a mixture of p19 and p42.

The p42 certainly induced a certain level of protection. However, as shown in Figures 6E and 6F. the protection conferred by the recombinant p19 of the invention was considerably better.

The hypothesis can be formulated that the improved protection results from secondary cleavage of p42 which is accompanied by revealing free cysteine which, as a result, forms intermolecular bridges giving rise to p19 multimers which are highly characteristic of this form in recombinant proteins of the three species tested

The numbers used to produce graphs (6A-6F) are given in **Figure 6G**

P. cynomolgi toque macaque vaccination test: second challenge infection of monkeys vaccinated with p19 alone and controls (Figures 8)

Six months later, with no other vaccination, the 3 macaques which received the p19 MSP-1 alone with FCA/FIA (Figure 6E) and the 3 macaques which received a saline solution containing Freund adjuvant (Figure 6B) and 2 new naive unvaccinated monkeys underwent a new challenge infection by injecting 1×10^6 red blood cells infected with *Plasmodium cynomolgi*. Protection was evaluated by determining parasitemia daily in all animals by examining Giemsa smears. The parasitemia were classified negative only after counting 400 smear fields The parasitemia were expressed as the percentage of parasitised red blood cells (the figures used to produce graphs 8A-C are given in **Figure 8D**). The six immunised animals which underwent challenge infection six months earlier had no detectable parasitemia except for 1 animal in each group which exhibited a parasitemia of 0.008% for 1 day (**Figures 8A and 8B**). The two naive controls exhibited a conventional parasitemia with a maximum of 0.8% and for 21 days (**Figure 8C**). Thus the 3 animals vaccinated with the MSP-1 p19 were also protected six months later than the 3 controls which exhibited a complete conventional infection after the first challenge infection, despite the absence of or a very slight parasitemia after the first challenge infection. These results suggest that the protection period for p19 is at least six months.

Vaccination test with p19 in association with alum in the _P. cynomolgi_ toque macaque system (Figures 9)

The previous positive protection results were obtained using complete (FCA) or incomplete (FIA) Freund adjuvant. However, the only adjuvant which is currently allowed in man is alum. For this reason, we carried out a vaccination test with *P. cynomolgi* MSP-1 p19 in the toque macaque in the presence of alum as the

adjuvant Six captured macaques were used as follows (1) 3 animals injected with 4 doses of 50 mg of recombinant *P. cynomolgi* MSP-1 p19 with 20 mg of alum. (2) 3 animals injected 4 times with physiological water and 10 mg of alum The injections were intramuscular at 4 week intervals. The challenge infection was made by injecting 2 x 10^5 red blood cells infected with *P. cynomolgi* 4 weeks after the last injection Protection was evaluated by daily determination of parasitemia in all animals by examining Giemsa smears. The parasitemia were classified negative only after counting 400 smear fields. Parasitemia were expressed as the percentage of parasitised red blood cells The results of this experiment were as follows 2 of the 3 macaques immunised with recombinant p19 with alum had about 30 times less total parasitemia during the infection period (**Figures 9A and 9B**) than the 3 control macaques immunised with physiological water and alum (**Figure 9D**) after the challenge infection The third macaque immunised with p19 (**Figure 9C**) was not very different from the controls For the vaccination test using *Plasmodium cynomolgi* p19 in the toque macaque. *macaca sinica*. described in Figure 9. the data used to produce the graphs (9A-9D) are given in (**Figure 9E**) While the results are a little less spectacular than the preceding results (Figures 6. 8). this is the first time that significant protection has been observed for recombinant MSP-1 with alum.

Figure 10: Vaccination test with a recombinant *Plasmodium falciparum* p19 in the squirrel monkey

Twenty *Saimiri sciureus guyanensis* (squirrel monkeys) of about 3 years old raised in captivity were used as follows: (1) 4 animals injected with 50 mg of soluble Pf MSP-1 p19 in the presence of Freund adjuvant as follows: 1st injection. 1:1 FCA/FIA: 2nd injection: 1 4 FCA/FIA: 3rd injection. FIA. These adjuvant compositions were then mixed with 1:1 antigen in PBS; (2) 2 control animals received Freund adjuvant as described for (1) with only PBS; (3) 4 animals injected with 50 mg of soluble Pf MSP-1 p19 in the presence of 10 mg of alum (Alu-Gel-S. Serva); (4) 2 control animals received 10 mg of alum with only PBS; (5) 4 animals injected with about 50-100 mg of GPI anchored Pf MSP-1 p19 reconstituted into liposom s as follows: 300 mmoles of cholesterol and 300

mmoles of phosphatidyl choline were vacuum dried and resuspended in 330 mM of N-octylglucoside in PBS with 1.4 mg of Pf MSP-1 p19. GPI. This solution had been dialysed against PBS with adsorbent Bio-Beads SM-2 (Bio-Rad) and the liposomes formed were concentrated by centrifuging and resuspended in PBS. The 1st injection was made with fresh liposomes kept at 4°C and the 2nd and 3rd injections were made with liposomes which had been frozen for preservation. (6) 2 animals injected with control liposomes made in the same way, in the absence of the p19. GPI antigen as described for (5). (7) 2 animals injected with physiological water. Three intramuscular injections were made at 4 week intervals. The challenge infection was made by injecting 1×10^{n} red blood cells infected with *Plasmodium falciparum*. Protection was evaluated by determining parasitemia daily in all animals by examining the Giemsa smears. Parasitemia were expressed as the percentage of parasitised red blood cells. The results of this vaccination test are shown in **Figures 10. A-G.**

The groups immunised with p19 in Freund adjuvant or liposome demonstrated similar parasitemia to the control groups after a challenge infection (one animal (number 29) vaccinated with p19 in Freund adjuvant died several days after challenge infection for reasons independent of vaccination (cardiac arrest)). Irregularities in administration of the antigen in these 2 groups (poor Freund emulsion, congealed liposomes) did not allow the significance of these results to be completely evaluated. In the alum group, 2 animals showed total parasitemia for the duration of the infection about 4 times less than the controls, 1 animal about 3 times less and 1 animal was similar to the controls. This experiment was a little difficult to interpret due to the variability in the controls, probably due to the strain of parasite used for the challenge infection which would not have been quite adapted to the non splenectomised *Saimiri* model developed only recently in Cayenne. However, the real effect with alum, although imperfect, is encouraging in that our antigens seem to be the only recombinant *P. falciparum* MSP-1 versions which currently have shown a certain effectiveness in combination with alum.

Vaccination test with a recombinant *Plasmodium falciparum* p19 in the squirrel monkey (same test as for Figures 10)

Monkeys bred in captivity were injected intramuscularly with 1 ml of inoculum twice at 4 week intervals as follows (1) 4 animals injected with 50 µg of soluble PfMSP1p19 in the presence of Freund adjuvant as follows 1st injection 1 1 FCA/FIA. 2nd injection 1 4 FCA/FIA. and mixed then 1 1 with the antigen in PBS. (2) 4 animals injected with 50 µg of soluble PfMSPp19 in the presence of 10 mg of alum. (3) 4 animals injected with about 50 µg of GPI anchored PfMSP1p19 reconstituted into liposomes composed of 1 1 molar cholesterol and phosphatidyl choline The animals were bled 17 days after the second injection

Red cells from a squirrel monkey with 30% parasitemia due to *P. falciparum* (with the mature forms in the majority) were washed with PBS and the residue was diluted 8 times in the presence of 2% SDS and 2% dithiothreitol and heated to 95° before being charged onto a polyacrylamide gel of 7 5% (separation gel) and 4% (stacking gel). After transfer to nitrocellulose. immunoblot analysis was carried out with antisera as follows. (1) pool of antisera of 4 monkeys vaccinated with soluble PfMSP1p19 in Freund adjuvant. twentieth dilution. (2) pool of antisera of 4 monkeys vaccinated with soluble PfMSP1p19 in alum adjuvant. twentieth dilution; (3) pool of antisera of 4 monkeys vaccinated with anchored PfMSP1p19 in liposomes. twentieth dilution; (4) monoclonal antibody. which reacts with a linear epitope of PfMSP1p19. 50 mg/ml. (5) SHI90 antisera pool originating from about twenty monkeys repeatedly infected with *P. falciparum* and which had become unaffectable by any subsequent infection with *P. falciparum*, five hundredth dilution; (6) antiserum pool of naive monkeys (never exposed to *P. falciparum*). twentieth dilution.

The results show that the 3 antiserum pools of monkeys vaccinated with PfMSP1p19 reacted strongly and specifically with very high molecular weight complexes (diffuse in the stacking gel) and present in parasite extracts containing more mature forms. These results support the hypothesis that a specific aggregate of PfMSP1p19 is present *in vivo* comprising epitopes which are reproduced in recombinant PfMSP1p19 molecules synthesised in the baculovirus system. in particular oligomeric forms thereof.

Diagnostics

The recombinant molecules PvMSP1p42 and PvMSP1p19 of the invention, derived from baculovirus, can and have been used to produce specific murine monoclonal antibodies. These antibodies, in combination with polyclonal anti-p42 antisera originating from another species such as the rabbit or goat can form the basis of a semi-quantitative diagnostic test for malaria which can distinguish between malaria due to *P. falciparum*, which can be fatal, and malaria due to *P. vivax*, which is generally not fatal. The principle of this test is to trap and quantify any MSP-1 molecule containing the p42 portion in the blood.

In this context, the advantages of the recombinant 42s, in particular partially deleted recombinant p42s, are as follows:

(i) they are both extremely well conserved in the same species and sufficiently divergent between different species to enable specific species reactants to be produced, under conditions in which antibodies derived from different *Plasmodiums*, in particular against *P. falciparum* and *P. vivax* can be produced which do not give rise to cross reactions,

(ii) since the recombinant p42 molecules derived from baculovirus appear to reproduce more of the native structure of the corresponding native proteins, the antibodies produced against these proteins will be well adapted to diagnostic use.

The microorganisms identified below have been deposited under Rule 6.1 of the Treaty of Budapest of <u>1st February 1996</u>, under the following registration numbers:

<u>Identification reference</u>	<u>Registration numbers</u>
PvMSP1p19A	I-1659
PvMSP1p19S	I-1660
PfMSP1p19A	I-1661
PfMSP1p19S	I-1662
PcMSP1p19S	I-1663

Figure 7 also illustrated these results It shows immunoblots produced on gel The first three gel tracks illustrate the *in vivo* response of monkeys to injections of p19 [(1) with Freund adjuvant. (2) with alum. (3) in the form of a liposome] and in particular the existence of high molecular weight complexes supporting the hypothesis of *in vivo* aggregation of p19 in the form of an oligomer. specific to the maturation stage (when p42 is cut into p19 and p33)

This vaccination test also comprised a third injection identical to the previous injections The injection with Freund adjuvant contained only FIA

Figure 7B The data for this Figure were derived from the squirrel monkey *P. falciparum I* vaccination test (Figure 10 below). The numbers correspond to the individual monkeys noted in Figure 10 The techniques and methods for this Figure were the same as fo Figure 7 except that the individual antiserum for each monkey was tested after three injections the day of the proof injection and the SHI antiserum was diluted by 1.250 The results show that the antiserum for 4 monkeys vaccinated with p19 and alum reacted strongly and specifically with very high molecular weight complexes while the monkeys of other groups vaccinated with p19 and Freund adjuvant or liposomes showed only a little reactivity with these complexes. Since the monkeys vaccinated with p19 and alum were also the best protected, this reactivity with the high molecular weight complexes appeared to indicate a protective effect. despite one monkey in the group not being protected with respect to the controls and that another was only partially protected.

The invention also, of course. concerns other applications. for example those described below with respect to certain of the examples. although these are not limiting in character.

Therapy

The recombinant molecules of the invention can be used to produce specific antib dies which can possibly be used by passive transfer for a therapy for severe malaria due to *P. falciparum* when there is a risk of death.

The invention also concerns the use of these antibodies, preferably fixed to a solid support (for example for affinity chromatography) for the purification of type p19 peptides initially contained in a mixture

Purification means bringing this mixture into contact with an antibody, dissociating the antigen-antibody complex and recovering the purified p19 type peptide.

When the following claims state that the less conserved portion or portions of region III is/are deleted from the p42, they are preferably regions containing at least 10 amino acids and in which the degree of conservation in *P. vivax*, *P. cynomolgi* and *P. falciparum* is less than 70% (less than seven out of ten amino acids identical when they are aligned).

The polyclonal and monoclonal antibodies of the present invention presented as recognising p42s are preferably those which more specifically recognise regions other than region IV, with the exclusion of region IV itself. Preferably, they recognise region I of p42.

The invention also concerns hybridomas secreting specific antibodies selectively recognising the p42 of a MSP-1 protein in the merozoite form of a *Plasmodium* type parasite which is infectious for man other than *Plasmodium vivax* and which does not recognise *Plasmodium vivax*.

In particular, these hybridomas secrete monoclonal antibodies which do not recognise *Plasmodium vivax* p42 or which specifically recognise *Plasmodium falciparum* p42.

The invention also concerns a hybridoma characterized in that it produces a monoclonal antibody which specifically recognises the p42 of *P. vivax* and of *P. cynomolgi*. A F10-3 hybridoma has been constructed from the X63 Ag8 653 myeloma producing IgG 2b/k recognising the p42 glycoprotein of *P. vivax*.

The invention also concerns vaccine compositions, also comprising mixtures of proteins or fragments, in particular mixtures of the type:

- *P. falciparum* p42 and *P. vivax* p42;
- *P. falciparum* p42 and *P. falciparum* p19;

- *P. vivax* p42 and *P. vivax* p19;
- *P. falciparum* p42, *P. falciparum* p19, <u>and</u> *P. vivax* p19 and *P. vivax* p42.

In all of the above compositions, the p42 is if necessary deprived of its most hypervariable regions.

As an example, the region which corresponds to that of the p19 fragment normally included in the p42 is itself partially deleted, this region comprising at least one of the two EGF regions normally contained in this p19.

It may also be deprived of region II, or even of the N-terminal region of region III or all of region III.

The invention is not limited to the production of human vaccine. It is also applicable to the production of veterinary vaccine compositions using the corresponding proteins or antigens derived from parasites which are infectious for mammals and products under the same conditions. It is known that infections of the same type, babesiosis, also appear in cattle, dogs and horses. One of the antigens of the Babesia species has a high conformational homology (in particular in the two EFG-like and cysteine-rich domains) and functional homology with a protein portion of MSP-1 [(36), (37) and (38)].

Examples of veterinary vaccines using a soluble antigen against such parasites have been described (39).

It goes without saying that the p42s used in these mixtures can also be modified as described in the foregoing when considered in isolation.

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CLAIMS

1. A recombinant protein in which the essential constituent polypeptide sequence is:
   - either that of a 42 kilodalton (p42) fragment of the surface protein I of the merozoite form (MSP-1 protein) of a *Plasmodium* type parasite which is infectious for man, from which region II and, if necessary, one or more parts of region III, in particular the less well conserved parts, have been deleted;
   - or that of a portion of that fragment which is also capable of inhibiting a parasitemia normally induced *in vivo* by the corresponding parasite;
   - or that of a peptide which is capable of inducing a cellular and/or humoral immunological response equivalent to that produced by said p42 fragment or said portion of that fragment; and

   said recombinant protein possibly comprising conformational epitopes which are unstable in a reducing medium and which constitute the majority of the epitopes recognised by human antiserums formed against the corresponding *Plasmodium*.

2. A recombinant protein according to claim 1, characterized in that its region which corresponds to that of the p19 fragment normally included in the p42 is itself partially deleted, this region including at least one of the two EGF regions normally contained in this p19.

3. A recombinant protein according to claim 2, characterized in that the molecular weight of said portion of p19 fragment is in the range 10 to 25 kDa, in particular in the range 10 to 15 kDa.

4. A recombinant protein according to claim 1, characterized in that it contains both the essential portions of the polypeptide sequence of region I and of region IV of the p42 and in that it is deprived of region II.

5. A recombinant protein according to claim 4, characterized in that the polypeptide sequence is that of the p42 from which region II has been deleted.

6. A recombinant protein according to claim 5, from which the N-terminal region of region III or all of region III has also been deleted

7. A recombinant protein according to any one of claims 1 to 6, characterized in that it also comprises a glycosylphosphatidylinositol (GPI) group of the type enabling the p19 fragment the sequence of which is normally included in that of the p42 to anchor to the host cell, in particular a eukaryote cell, preferably a cell of an insect infectable by a baculovirus, in which said recombinant protein is expressed.

8. A recombinant protein according to any one of claims 1 to 7, characterized in that it is deprived of the extremely hydrophobic C-terminal portion which intervenes in induction of anchoring of said recombinant protein to the cell membrane of the host in which it is expressed, in particular in a eukaryote cell, preferably a cell of an insect infectable by a baculovirus.

9. A recombinant protein according to claim 8, characterized in that it is hydrosoluble.

10. A recombinant protein according to any one of claims 1 to 9, characterized in that it contains said partially deleted p42 sequence of the MSP-1 protein of *Plasmodium falciparum* or said portion of the corresponding fragment.

11. A recombinant protein according to any one of claims 1 to 9, characterized in that it contains said partially deleted p42 sequence of the MSP-1 protein of *Plasmodium cynomolgi* or said portion of the corresponding fragment.

12. A recombinant protein according to any one of claims 1 to 9, characterized in that it contains said partially deleted p42 sequence of the MSP-1 protein of *Plasmodium vivax* or said portion of the corresponding fragment.

13. A recombinant protein according to any one of claims 1 to 12, characterized in that it is conjugated to a carrier molecule for use in the production of vaccines.

14. A vaccination composition against a *Plasmodium* type parasite which is infectious for man, containing as an active principle a recombinant protein according to any one of claims 1 to 13.

15 An antibody specifically recognising the p42 of a MSP-1 protein of the merozoite form of a *Plasmodium* type parasite which is infectious for man other than *Plasmodium vivax* and which does not recognise *Plasmodium vivax*

16. A specific antibody according to claim 15, characterized in that it does not recognise *Plasmodium vivax*.

17 A specific antibody according to claim 15, characterized in that it specifically recognises the p42 of *P. falciparum*.

18. A specific antibody according to claim 15, characterized in that it specifically recognises the p42 of *P. vivax*.

19 A differential diagnostic process to distinguish between a parasitic infection due to *P. vivax* and a parasitic infection due to another *Plasmodium*, characterized by bringing a biological sample infected with *Plasmodium* into contact with an antibody according to claim 18 and with an antibody according to claim 16 or claim 17, and detecting the production or no production of an immunological reaction depending on the case

20. A recombinant baculovirus type modified vector containing, under the control of a promoter contained in the vector and able to be recognised by cells transfectable by said vector, a first nucleotide sequence coding for a signal peptide which can be used in a baculovirus system, characterized by a second sequence downstream of the first, also under the control of said promoter, of which at least a portion codes for a peptide sequence:

- either that of a 42 kilodalton (p42) C-terminal fragment of the surface protein 1 of the merozoite form (MSP-1 protein) of a *Plasmodium* type parasite which is infectious for man, from which, if necessary, the region II and, if necessary also, one or more parts of region III have been deleted, in particular the less well conserved portions thereof;
- or that of a portion of that peptide fragment provided that the expression product from the second sequence in a baculovirus system is also capable of inhibiting a parasitemia normally induced *in vivo* by the corresponding parasite;

- or that of a peptide which is capable of inducing a cellular and/or humoral immunological response equivalent to that produced by said peptide fragment p42 or said peptide fragment portion; and

said nucleotide sequence also having a G and C content in the range 40% to 60%, preferably at least 50%, of the totality of nucleotides from which it is constituted.

21. A modified vector according to claim 20, characterized in that the said second polypeptide sequence is in accordance with that defined in any one of claims 2 to 9.

22. A modified vector according to claim 20, characterized in that the second nucleotide sequence is a synthetic sequence

23. A modified vector according to any one of claims 20 to 22, characterized in that the first nucleotide sequence codes for a signal peptide from *Plasmodium vivax* and normally associated with the *Plasmodium* MSP-1 protein.

24. A modified vector according to any one of claims 20 to 23, characterized in that the second nucleotide sequence is deprived at its 3' terminal end of the hydrophobic C-terminal end sequence which is implicated in induction of anchoring said recombinant protein to the cell membrane of the host in which it is expressed, in particular in a cell of an insect infectable by a baculovirus.

25. A modified vector according to any one of claims 20 to 24, characterized in that it consists of a modified baculovirus.

26. An organism, in particular an Sf9 type insect cell, transfectable and transfected by the modified vector according to any one of claims 20 to 24.

27. A synthetic DNA containing a first nucleotide sequence for which at least a portion codes for a peptide sequence:

- either of a 42 kilodalton (p42) C-terminal fragment of the surface protein 1 of the merozoite form (MSP-1 protein) of *Plasmodium falciparum*, which is infectious for man, from which, if necessary, region II and, also if necessary, one or more parts of region III, in particular the less well conserved parts thereof, have been deleted;

- or of a portion of that peptide fragment provided that the expression product of said DNA in a baculovirus system is also capable of inhibiting a parasitemia normally induced *in vivo* by the corresponding parasite.
- or of a peptide capable of inducing a cellular and/or humoral type immunological response equivalent to that produced by said p42 peptide fragment or said portion of that fragment: and

said nucleotide sequence also having a G and C nucleotide content in the range 40% to 60%, preferably at least 50%, of the totality of nucleotides from which said synthetic DNA is constituted.

28. A synthetic DNA sequence according to claim 27, characterized in that its first nucleotide sequence is deprived at its 3' terminal end of the sequence coding for the hydrophobic C-terminal end region normally implicated in inducing anchoring of the p19 protein the sequence of which is included in that of the p42, to the cell membrane of the host in which it is expressed, in particular in a cell of an insect infectable by a baculovirus.

29. A synthetic DNA sequence according to claim 27 or claim 28, characterized in that the first nucleotide sequence is preceded by a signal nucleotide sequence coding for a signal peptide normally associated with a *Plasmodium* MSP-1 protein, homologous or heterologous relative to the principal sequence.

30. A synthetic DNA sequence according to claim 29, characterized in that the signal sequence originates from *P. vivax*.

31. A synthetic DNA according to any one of claims 27 to 30, characterized in that said first nucleotide sequence includes a 3'-terminal sequence coding for a polypeptide cell membrane anchoring region, said anchoring region fixing the expressed recombinant protein to the surface of the membrane of the host cell transformed with a vector containing said synthetic DNA, said 3' sequence being homologous to that of the principal nucleotide sequence, or heterologous, in particular that from *P. vivax*.

32. A synthetic DNA according to claim 31, characterized in that the 3'-terminal sequence originates from *P. vivax*.

33 A synthetic DNA sequence according to any one of claims 27 to 31, characterized in that it is deprived of said 3'-terminal sequence

34 A hybridoma secreting monoclonal antibodies having the specifications of the antibodies of any one of claims 15 to 18

35 A process for separating a p42 peptide with a given specificity from a mixture of peptides, characterized by bringing said peptide mixture into contact with a corresponding antibody, in accordance with any one of claims 15 to 18, preferably already fixed on an insoluble support, by subsequently dissociating the antigen-antibody compound formed and by recovering the purified p42 peptide.

36. Use of a protein according to any one of claims 1 to 12 to prepare an immunogen composition which can induce an immune response against a *Plasmodium* infection.

37. A vaccine composition comprising, as active principles, a mixture of a protein according to any one of claims 1 to 13 and either the corresponding p19, or another recombinant p42 or p19 type protein, originating from a parasite homologous with that from which said protein originates

38. A vaccine composition according to claim 37, characterized in that the mixture of active principles is selected from the following mixtures

- *P. falciparum* p42 and *P. vivax* p42;
- *P. falciparum* p42 and *P. falciparum* p19;
- *P. vivax* p42 and *P. vivax* p19;
- *P. falciparum* p42 and *P. falciparum* p19, <u>and</u> *P. vivax* p19 and *P. vivax* p42,

the p42 if necessary being deprived of its most hypervariable regions.

39. A hybridoma according to claim 34, characterized in that it has been deposited at the CNCM with registration number I-1846, on the 14th February 1997.

FIGURE 1A

```
        E   F   N   I   S   Q   H   Q   C   V   K   K   Q   C   P   E   N
Bac 19  GAA TTC AAC ATC TCG CAG CAC CAA TGC GTG AAA AAA CAA TGT CCC GAG AAC

PF 19           AAC ATT TCA CAA CAC CAA TGC GTA AAA AAA CAA TGT CCA GAA AAT

        S   G   C   F   R   H   L   D   E   R   E   E   C   K   C   L   L
Bac 19  TCT GGC TGT TTC AGA CAC TTG GAC GAG AGA GAG GAG TGT AAA TGT CTG CTG

PF 19   TCT GGA TGT TTC AGA CAT TTA GAT GAA AGA GAA GAA TGT AAA TGT TTA TTA

        N   Y   K   Q   E   G   D   K   C   V   E   N   P   N   P   T   C
Bac 19  AAC TAC AAA CAG GAG GGC GAC AAG TGC GTG GAG AAC CCC AAC CCG ACC TGT

PF 19   AAT TAC AAA CAA GAA GGT GAT AAA TGT GTT GAA AAT CCA AAT CCT ACT TGT

        N   E   N   N   G   G   C   D   A   D   A   K   C   T   E   E   D
Bac 19  AAC GAG AAC AAC GGC GGC TGT GAC GCA GAC GCC AAA TGC ACC GAG GAG GAC

PF 19   AAC GAA AAT AAT GGT GGA TGT GAT GCA GAT GCC AAA TGT ACC GAA GAA GAT

        S   G   S   N   G   K   K   I   T   C   E   C   T   K   P   D   S
Bac 19  TCG GGC AGC AAC GGC AAG AAA ATC ACG TGT GAG TGT ACC AAA CCC GAC TCG

PF 19   TCA GGT AGC AAC GGA AAG AAA ATC ACA TGT GAA TGT ACT AAA CCT GAT TCT

        Y   P   L   F   D   G   I   F   C   S   *   *
Bac 19  TAC CCG CTG TTC GAC GGC ATC TTC TGC AGC TAA TAA

PF 19   TAT CCA CTT TTC GAT GGT ATT TTC TGC AGT
```

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FIGURE 1B

```
Site Eco RI
         E   F   N   I   S   Q   H   Q   C   V   K   K   Q   C   P   E   N
Bac 19   GAA_TTC AAC ATC TCG CAG CAC CAA TGC GTG AAA AAA CAA TGT CCC GAG AAC
PF 19            AAC ATT TCA CAA CAC CAA TGC GTA AAA AAA CAA TGT CCA GAA AAT

         S   G   C   F   R   H   L   D   E   R   E   E   C   K   C   L   L
Bac 19   TCT GGC TGT TTC AGA CAC TTG GAC GAG AGA GAG GAG TGT AAA TGT CTG CTG
PF 19    TCT GGA TGT TTC AGA CAT TTA GAT GAA AGA GAA GAA TGT AAA TGT TTA TTA

         N   Y   K   Q   E   G   D   K   C   V   E   N   P   N   P   T   C
Bac 19   AAC TAC AAA CAG GAG GGC GAC AAG TGC GTG GAG AAC CCC AAC CCG ACC TGT
PF 19    AAT TAC AAA CAA GAA GGT GAT AAA TGT GTT GAA AAT CCA AAT CCT ACT TGT

         N   E   N   N   G   G   C   D   A   D   A   K   C   T   E   E   D
Bac 19   AAC GAG AAC AAC GGC GGC TGT GAC GCA GAC GCC AAA TGC ACC GAG GAG GAC
PF 19    AAC GAA AAT AAT GGT GGA TGT GAT GCA GAT GCC AAA TGT ACC GAA GAA GAT

         S   G   S   N   G   K   K   I   T   C   E   C   T   K   P   D   S
Bac 19   TCG GGC AGC AAC GGC AAG AAA ATC ACG TGT GAG TGT ACC AAA CCC GAC TCG
PF 19    TCA GGT AGC AAC GGA AAG AAA ATC ACA TGT GAA TGT ACT AAA CCT GAT TCT

         Y   P   L   F   D   G   I   F   C   S   S   S   N   F   L   G   I
Bac 19   TAC CCG CTG TTC GAC GGC ATC TTC TGC AGC TCC TCT AAC TTC TTG GGC ATC
PF 19    TAT CCA CTT TTC GAT GGT ATT TTC TGC AGT TCC TCT AAC TTC TTA GGA ATA

         S   F   L   L   I   L   H   L   I   L   Y   S   F   I   *   *
Bac 19   TCG TTC TTG TTG ATC CTC ATG TTG ATC TTG TAC AGC TTC ATT TAA TAA
PF 19    TCA TTC TTA TTA ATA CTC ATG TTA ATA TTA TAC AGT TTC ATT
```

FIGURE 1C

```
ATG AAG GCG CTA CTC TTT TTG TTC TCT TTC ATT TTT TTC GTT ACC AAA TGT
 M   K   A   L   L   F   L   F   S   F   I   F   F   V   T   K   C

CAA TGT GAA ACA GAA AGT TAT AAG CAG CTT GTA GCC AAC GTG GAC GAA TTC
 Q   C   E   T   E   S   Y   K   Q   L   V   A   N   V   D   E   F

AAC ATC TCG CAG CAC CAA TGC GTG AAA AAA CAA TGT CCC GAG AAC TCT GGC
 N   I   S   Q   H   Q   C   V   K   K   Q   C   P   E   N   S   G

TGT TTC AGA CAC TTG GAC GAG AGA GAG GAG TGT AAA TGT CTG CTG AAC TAC
 C   F   R   H   L   D   E   R   E   E   C   K   C   L   L   N   Y

AAA CAG GAG GGC GAC AAG TGC GTG GAG AAC CCC AAC CCG ACC TGT AAC GAG
 K   Q   E   G   D   K   C   V   E   N   P   N   P   T   C   N   E

AAC AAC GGC GGC TGT GAC GCA GAC GCC AAA TGC ACC GAG GAG GAC TCG GGC
 N   N   G   G   C   D   A   D   A   K   C   T   E   E   D   S   G

AGC AAC GGC AAG AAA ATC ACG TGT GAG TGT ACC AAA CCC GAC TCG TAC CCG
 S   N   G   K   K   I   T   C   E   C   T   K   P   D   S   Y   P

CTG TTC GAC GGC ATC TTC TGC AGC TAA TAA
 L   F   D   G   I   F   C   S   *   *
```

FIGURE 1D

```
GAA ACA GAA AGT TAT AAG CAG CTT GTA GCC AAC GTG GAC GAA TTC
 E   T   E   S   Y   K   Q   L   V   A   N   V   D   E   F

AAC ATC TCG CAG CAC CAA TGC GTG AAA AAA CAA TGT CCC GAG AAC TCT GGC
 N   I   S   Q   H   Q   C   V   K   K   Q   C   P   E   N   S   G

TGT TTC AGA CAC TTG GAC GAG AGA GAG GAG TGT AAA TGT CTG CTG AAC TAC
 C   F   R   H   L   D   E   R   E   E   C   K   C   L   L   N   Y

AAA CAG GAG GGC GAC AAG TGC GTG GAG AAC CCC AAC CCG ACC TGT AAC GAG
 K   Q   E   G   D   K   C   V   E   N   P   N   P   T   C   N   E

AAC AAC GGC GGC TGT GAC GCA GAC GCC AAA TGC ACC GAG GAG GAC TCG GGC
 N   N   G   G   C   D   A   D   A   K   C   T   E   E   D   S   G

AGC AAC GGC AAG AAA ATC ACG TGT GAG TGT ACC AAA CCC GAC TCG TAC CCG
 S   N   G   K   K   I   T   C   E   C   T   K   P   D   S   Y   P

CTG TTC GAC GGC ATC TTC TGC AGC TAA TAA
 L   F   D   G   I   F   C   S   *   *
```

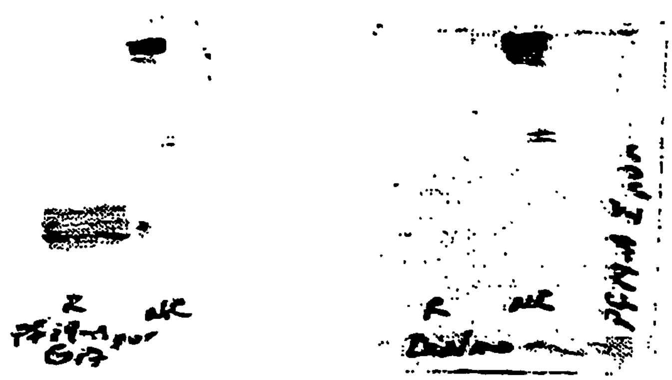


FIGURE 2

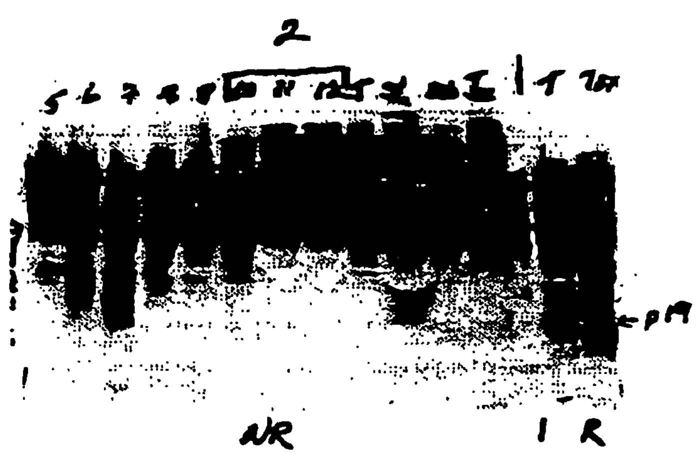


FIGURE 3

```
A        1
cynomolgi        DOVTTGEAES EAPEIIVPOG INEYDVVYIK PLAGMYKTIK KPLENIIVNAL
vivax (Belem)    DOVTTGEAES EAPEILVPAG ISDYDVVYLK PLAGMYKTIK KOLENIIVNAF
vivax (Sal I)    DOVTTGEAES EAPEILVPAG ISDYDVVYLK PLAGMYKTIK KOLENIIVNAF
Consensus        DOVTTGEAES EAPEI·VP·G I··YDVVY·K PLAGMYKTIK K·LENIIVNA·
                             ▲
                 51          42                   REGION I
cynomolgi        NTNIIDMLDS ALKKANYFLD VLNSDLNPYS IPHSGEYIIK DPYKLLDLE.
vivax (Belem)    NTNIIDMLDS ALKKANYFLE VLNSDLNPFK YSPSGEYIIK DPYKLLDLEK
vivax (Sal I)    NTNIIDMLDS ALKKANYFLE VLNSDLNPFK YSSSGEYIIK DPYKLLDLEK
Consensus        NTNI·DMLDS ALKKANYFL· VLNSDLNP·· ···SGEYIIK DPYKLLDLE·

                 101
cynomolgi        KKKLIGSYKY IGASVDHDMV TANDGLAYYQ KMGDLYKKHL
vivax (Belem)    KKKLIGSYKY IGASIDHDLA TANDGVTYYN KMGELYKTHL
vivax (Sal I)    KKKLIGSYKY IGASIDMDLA TANDGVTYYN KMGELYKTHL
Consensus        KKKL·GSYKY IGAS·D·D·· TANDG··YY· KMG·LYK·HL

                 141▶                REGION II
cynomolgi        DEVNACIKEV EANINKHDEE IKKIGSEASH ANDKNCLNAK
vivax (Belem)    TAVNEEVKKV EADIKAEDDK IKKIGGDSIH TTEKICSMAK
vivax (Sal 1)    DGVKTEIKKV EDDIKHDDEE LKKLDNVASD DSKKNEFIAK
Consensus        ··V····K·V E··I···D·· ·KK·G····· ···K····AK

                 181                 REGION III
cynomolgi        KEELQKYLPF LSSIQKEYST LVNKVHSYTD TLKKIINNCQ IEKKETETIV
vivax (Belem)    KAELEKYLPF LNSLQKEYES LVSKVNTYTD NLKKVINNCQ LEKKEAEITV
vivax (Sal I)    KAELEKYLPF LNSLQKEYES LVSKVNTYTD NLKKVINNCQ LEKKEAEITV
Consensus        K·EL·KYLPF L·S·QKEY·· LV·KV··YTD ·LKK·INNCQ ·EKKE·E··V
```

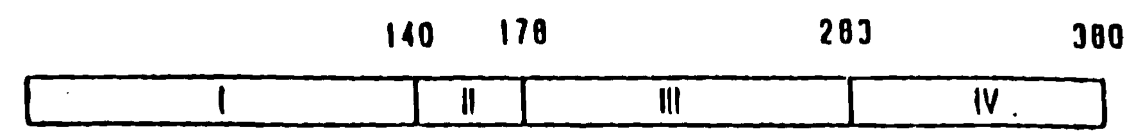
FIGURE 4(1)

FIGURE 4(2)

```
                231
cynomolgi       NKLEDYSKMD EELDVYKQSK KEDDVKSSGL LEKLMNSKLI NQEESKKALS
vivax (Belem)   KKLQDYNKMD EKLEEYKKSE KKNEVKSSGL LEKLMKSKLI KENESKEILS
vivax (Sal I)   KKLQDYNKMD EKLEEYKKSE KKNEVKSSGL LEKLMKSKLI KENESKEILS
Consensus       ·KL·DY·KMD E·L··YK·S· K···VKSSGL LEKLM·SKLI ···ESK··LS

                281
cynomolgi       ELLNVQTQML NMSSEHRCID TNVPENAACY RYLDGTEEWR CLLYFKEDAG
vivax (Belem)   QLLNVQTQLL TMSSEHTCID TNVPDNAACY RYLDGTEEWR CLLTFKEEGG
vivax (Sal I)   QLLNVQTQLL TMSSEHTCID TNVPDNAACY RYLDGTEEWR CLLTFKEEGG
Consensus       ·LLNVQTQ·L ·MSSEH·CID TNVP·NAACY RYLDGTEEWR CLL·FKE··G
                  19
                331                         REGION IV
cynomolgi       KCVPAPNMTC KDKNGGCAPE AECKMNDKNE IVCKCTKEGS EPLFEGVFCS
vivax (Belem)   KCVPASNVTC KDNNGGCAPE AECKMTDSNK IVCKCTKEGS EPLFEGVFCS
vivax (Sal I)   KCVPASNVTC KDNNGGCAPE AECKMTDSNK IVCKCTKEGS EPLFEGVFCS
Consensus       KCVPA·N·TC KD·NGGCAPE AECKM·D·N· IVCKCTKEGS EPLFEGVFCS
```

B



	I	II	III	IV
VIVAX (Belem):VIVAX (Sal-I)	98%	37%	100%	100%
CYNOMOLGI : VIVAX	84%	47%	69%	86%

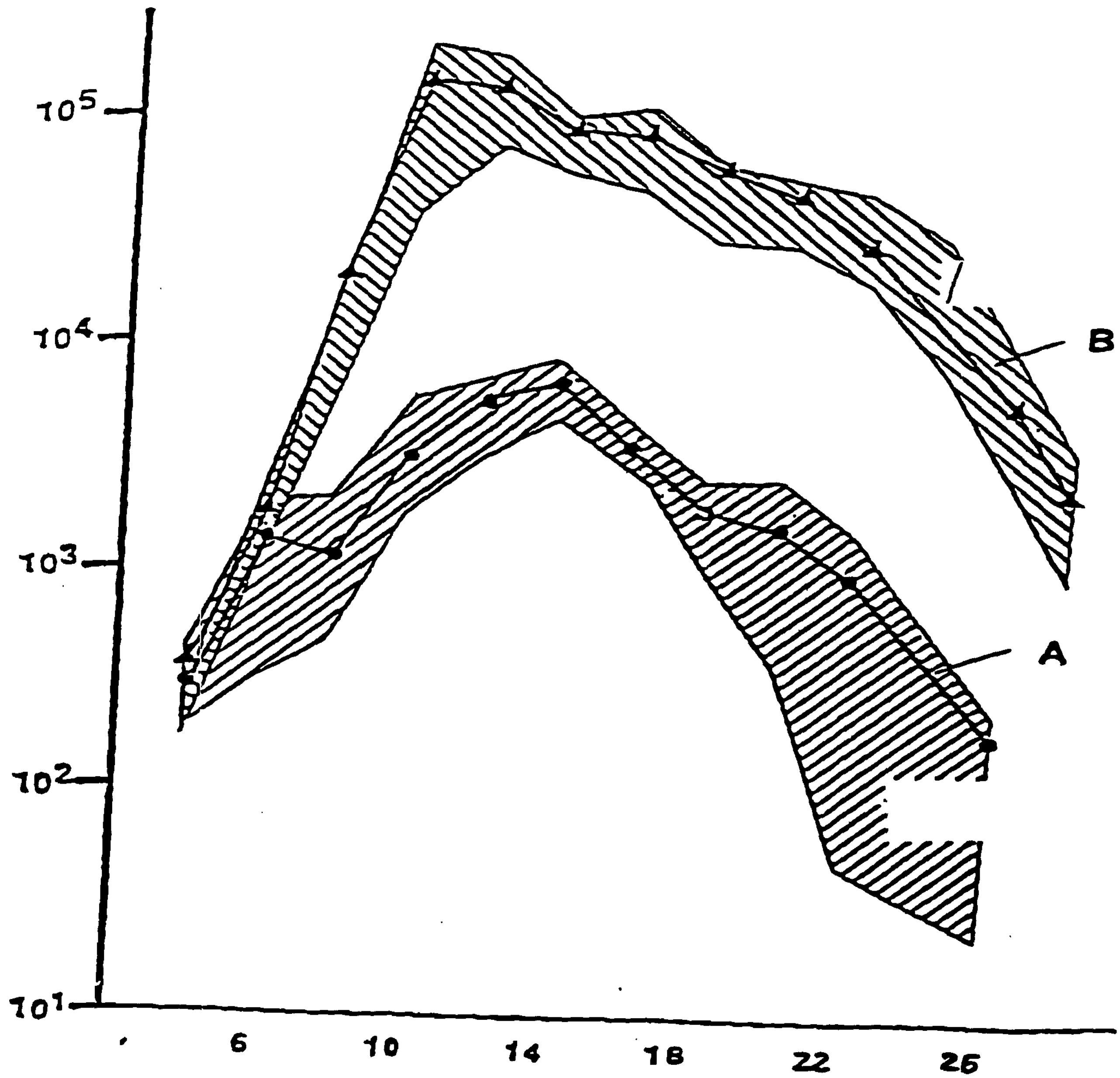


FIGURE 5

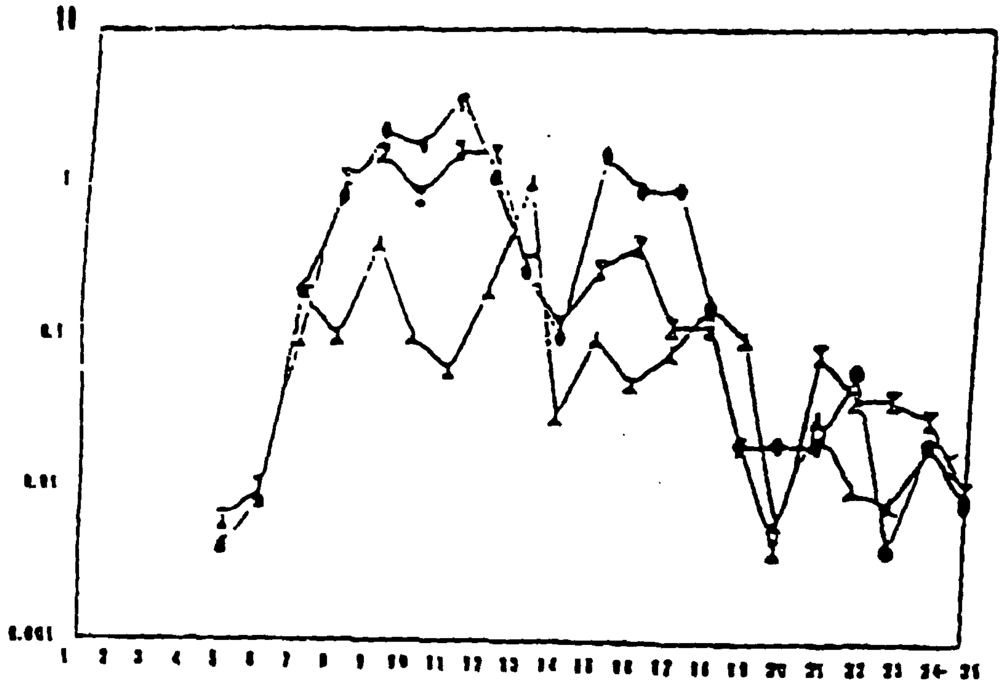


FIGURE 6A

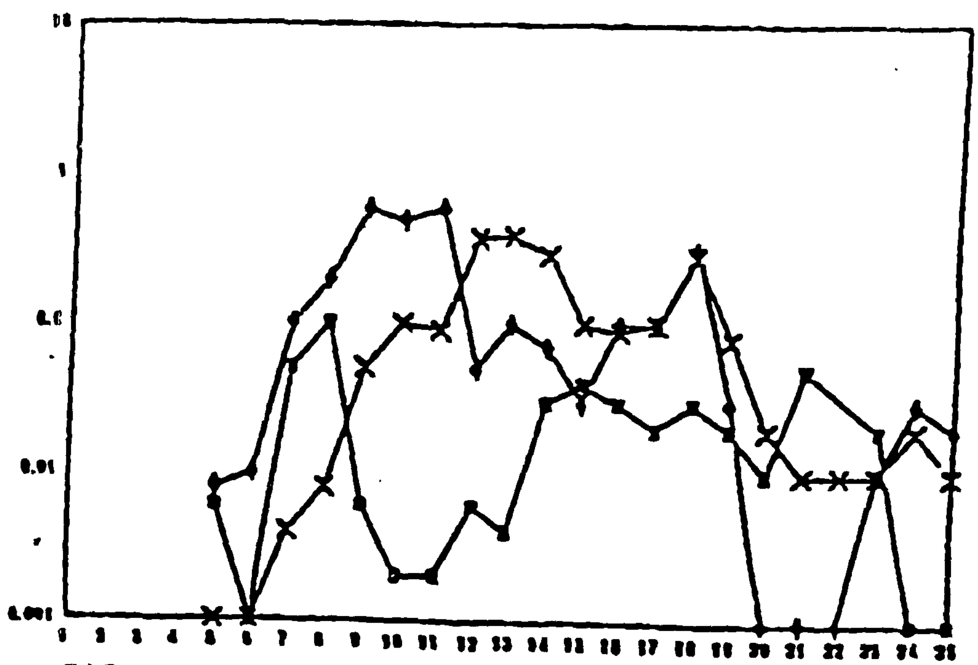


FIGURE 6B

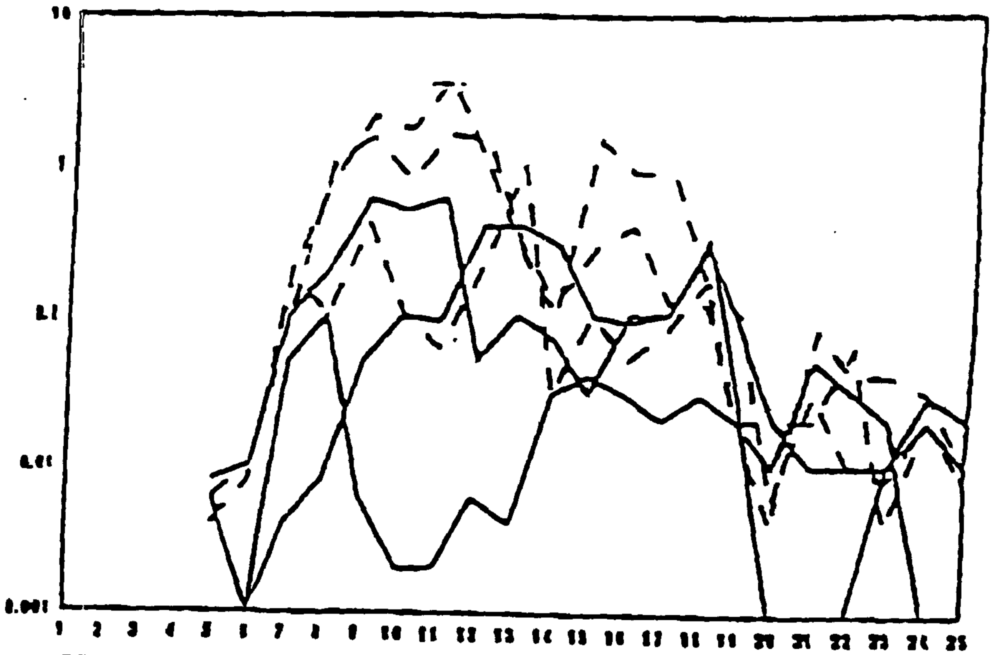


FIGURE 6C

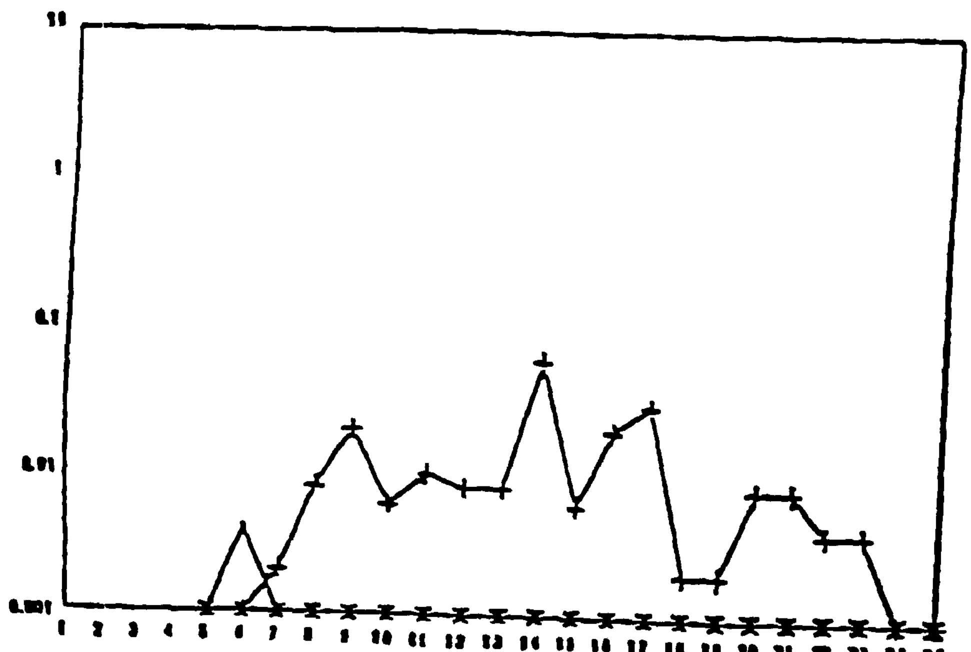


FIGURE 6D

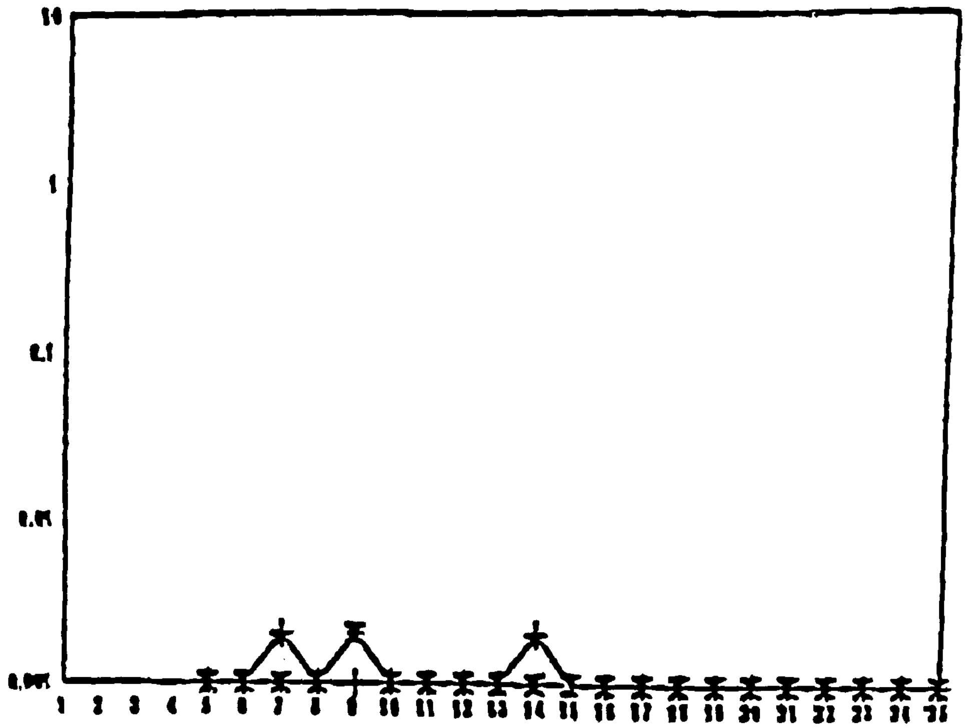
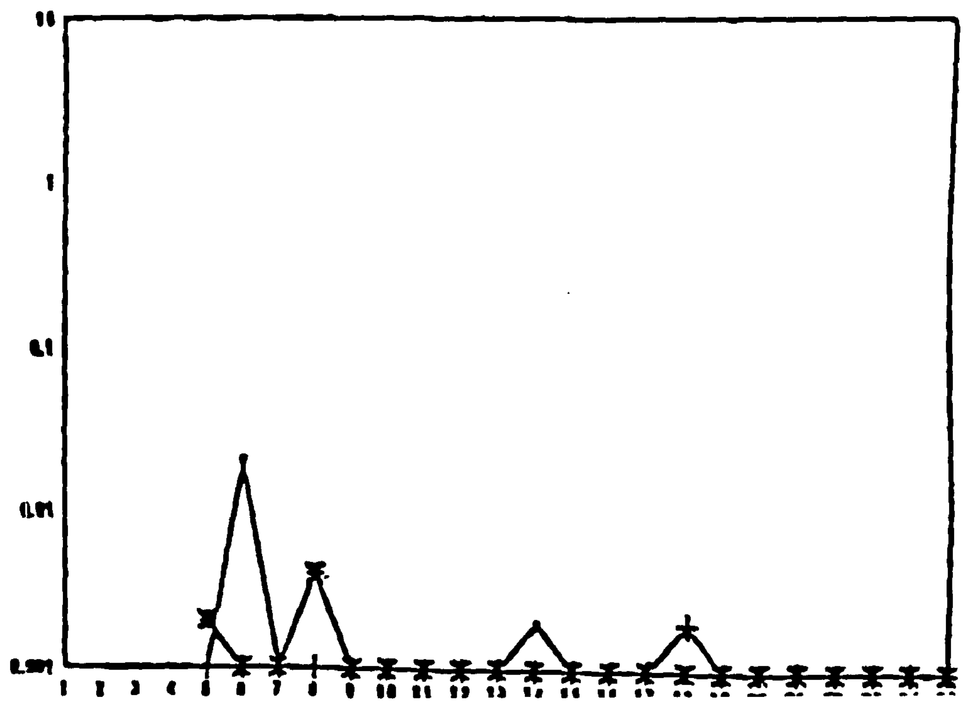


FIGURE 6E



Vaccination test: recombinant MSP-1 (p42 and p19) from *Plasmodium cynomolgi* in the *Macaca sinica* toque macaque

	Year	95	95	95	95	95	95	95	95	95	95	95	95	95	95
	Month	7	7	7	7	7	7	7	7	7	7	7	7	7	8
	Day	19	20	21	22	23	24	25	26	27	28	29	30	31	1
	Days post-infection	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Vaccination p42	T434	-	.004	-	-	-	-	-	-	-	-	-	-	-	-
	T435	-	-	.002	.008	.02	.006	.01	.008	.008	.06	.006	.02	.03	.002
	T428	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vaccination p19	T429	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	T426	-	-	.002	-	-	-	-	-	-	.002	-	-	-	-
	T427	-	-	-	-	.002	-	-	-	-	-	-	-	-	-
Vaccination p42+p19	T430	-	.01	-	-	-	-	-	-	-	.002	-	-	-	-
	T431	-	-	-	-	-	-	-	-	-	-	-	-	-	.002
	T433	.002	-	-	.004	-	-	-	-	-	-	-	-	-	-
Controls	T425	.006	-	.05	0.1	.006	.002	.002	.006	.004	.01	.04	.03	.02	.03
Physiological water	T436	-	-	.004	.008	0.05	0.1	0.09	0.39	0.4	0.3	0.1	0.09	0.1	.3
FCA/FIA	T438	.008	0.01	0.1	0.2	0.6	0.5	0.6	0.05	0.1	0.07	0.03	0.1	0.1	.32
Controls	T437	.004	.008	0.2	0.1	0.4	0.1	0.06	0.2	1.0	0.03	0.1	0.05	0.08	.17
Non vaccinated	T440	.006	.01	0.1	1.04	1.5	1.8	1.6	1.5	0.3	0.12	0.28	0.4	0.12	.12
	T441	.004	.008	0.2	0.8	2.1	1.7	3.8	1.04	0.27	0.1	1.5	0.9	0.9	.16

- = Absence of parasites in 400 microscopic fields

FIGURE 6G(1)

Vaccination test: recombinant MSP-1 (p42 and p19) from *Plasmodium cynomolgi* in the *Macaca sinica* toque macaque

Year		95	95	95	95	95	95	95	95	95	95	95	95	95	95
Month		8	8	8	8	8	8	8	8	8	8	8	8	8	8
Day		2	3	4	5	6	7	8	9	10	11	12	13	14	15
Days post-infection		19	20	21	22	23	24	25	26	27	28	29	30	31	32
Vaccination p42	T434	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	T435	.002	.008	.008	.004	.004	-	-	-	-	-	-	-	-	-
	T428	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vaccination p19	T429	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	T426	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	T427	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vaccination p42+p19	T430	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	T431	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	T433	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Controls Physiological water FCA/FIA	T425	.02	.01	.05	ND	.02	-	-	.004	.004	.01	.06	.04	.03	.008
	T436	.08	.02	.01	.01	.01	.02	.01	.01	.008	.02	.004	.008	.004	.004
	T438	.03	-	-	-	.01	.03	.02	.04	.01	.05	.004	.008	-	.004
Controls Non vaccinated	T437	.1	.006	.03	.01	.008	.02	.01	.008	.01	.01	.008	.01	-	-
	T440	.02	.004	.08	.04	.04	.03	.01	.01	.04	.02	.005	.03	.004	.004
	T441	.02	.02	.02	.06	.004	.02	.008	.01	.008	.004	.004	.008	.008	.004

- = Absence of parasites in 400 microscopic fields

FIGURE 6G(2)

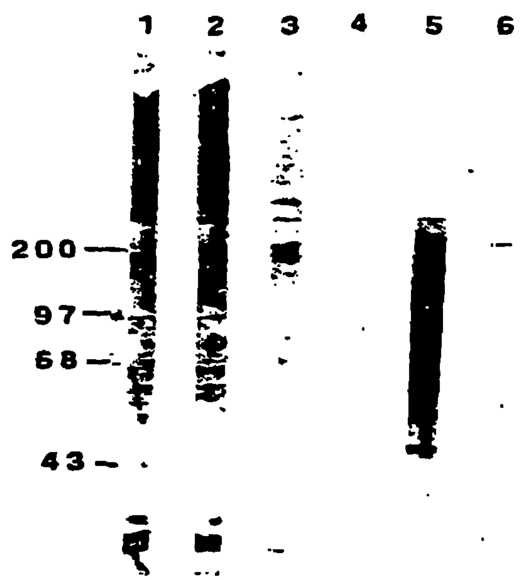


FIGURE 7 A

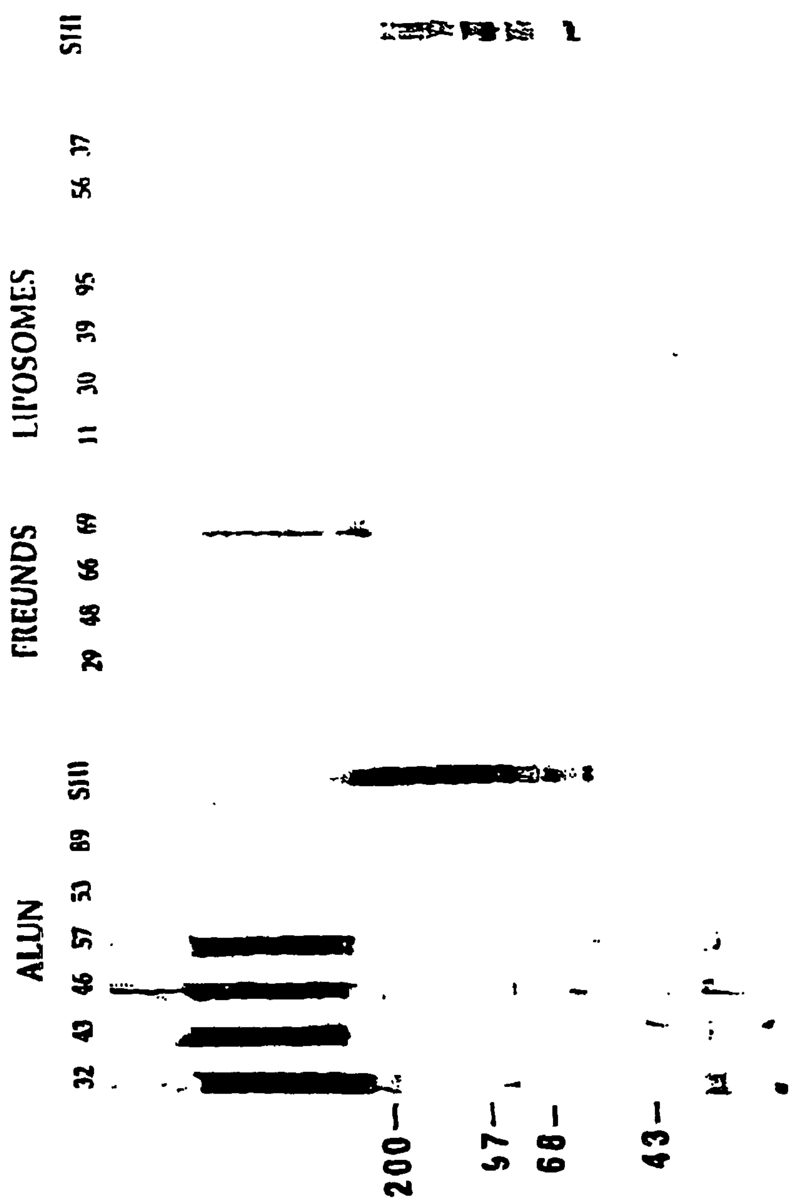

ALUN
32 43 46 57 53 89 SIII
FREUNDS
29 48 66 69
LIPOSOMES
11 30 39 95 56 37 SIII
200
97
68
43

FIGURE 7B

Vaccination test: recombinant MSP-1 (p19) of *Plasmodium cynomolgi* in the *Macaca sinica* toque macaque; second challenge infection

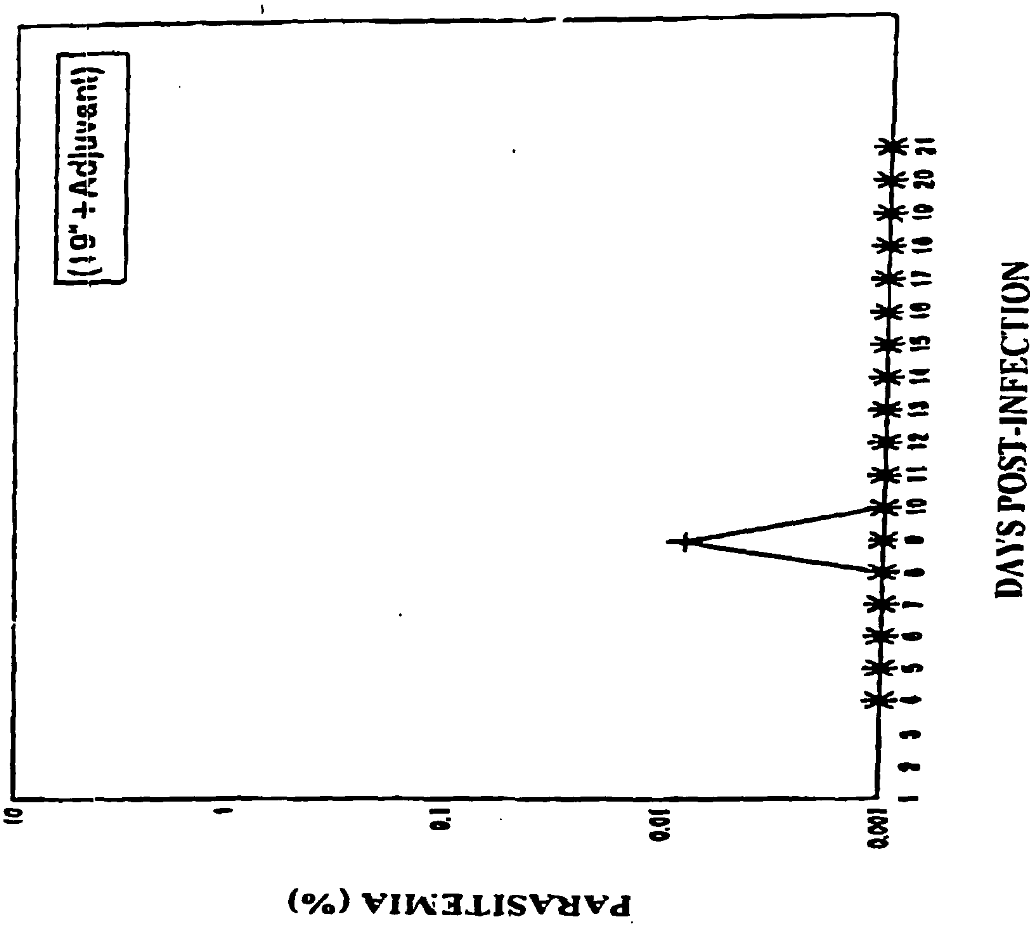


FIGURE 8A

Vaccination test: recombinant MSP-1 (p19) of *Plasmodium cynomolgi* in the *Macaca sinica* toque macaque; second challenge infection

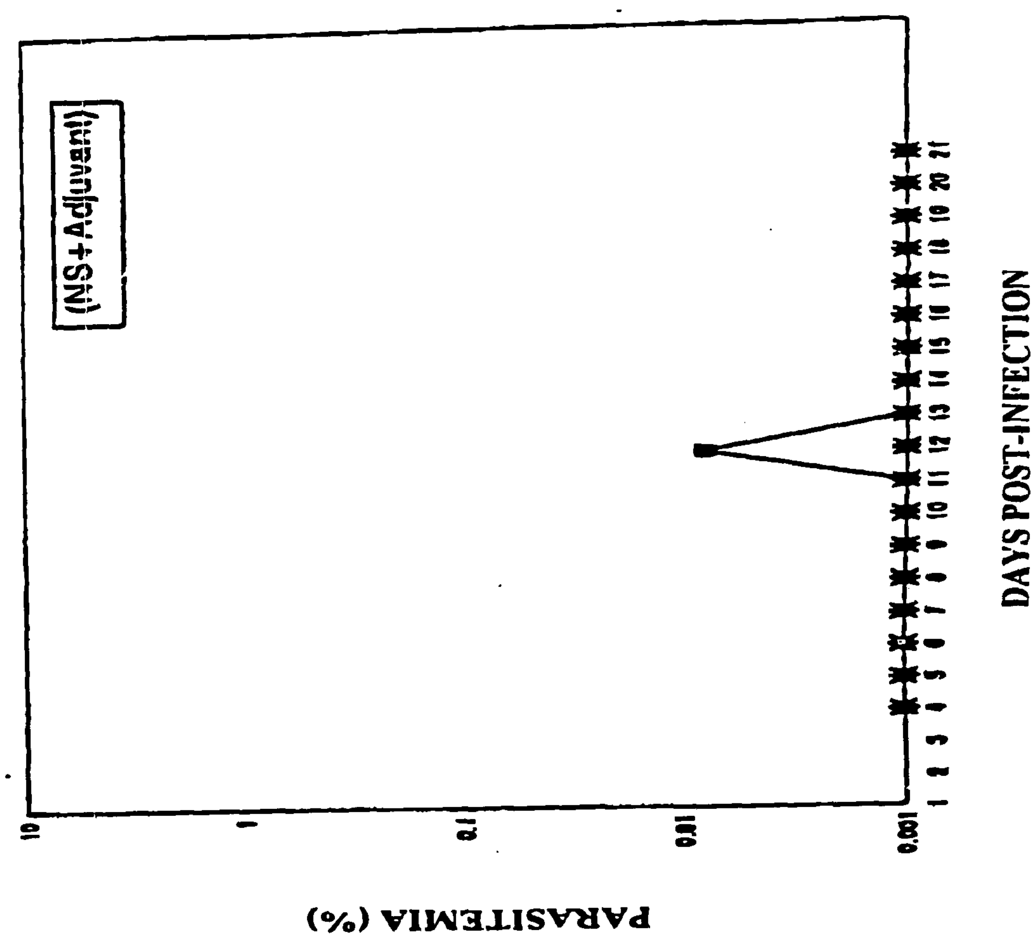


FIGURE 8B

REPLACEMENT PAGE (RULE 26)

Vaccination test: recombinant MSP-1 (p19) of *Plasmodium cynomolgi* in the *Macaca sinica* toque macaque; second challenge infection

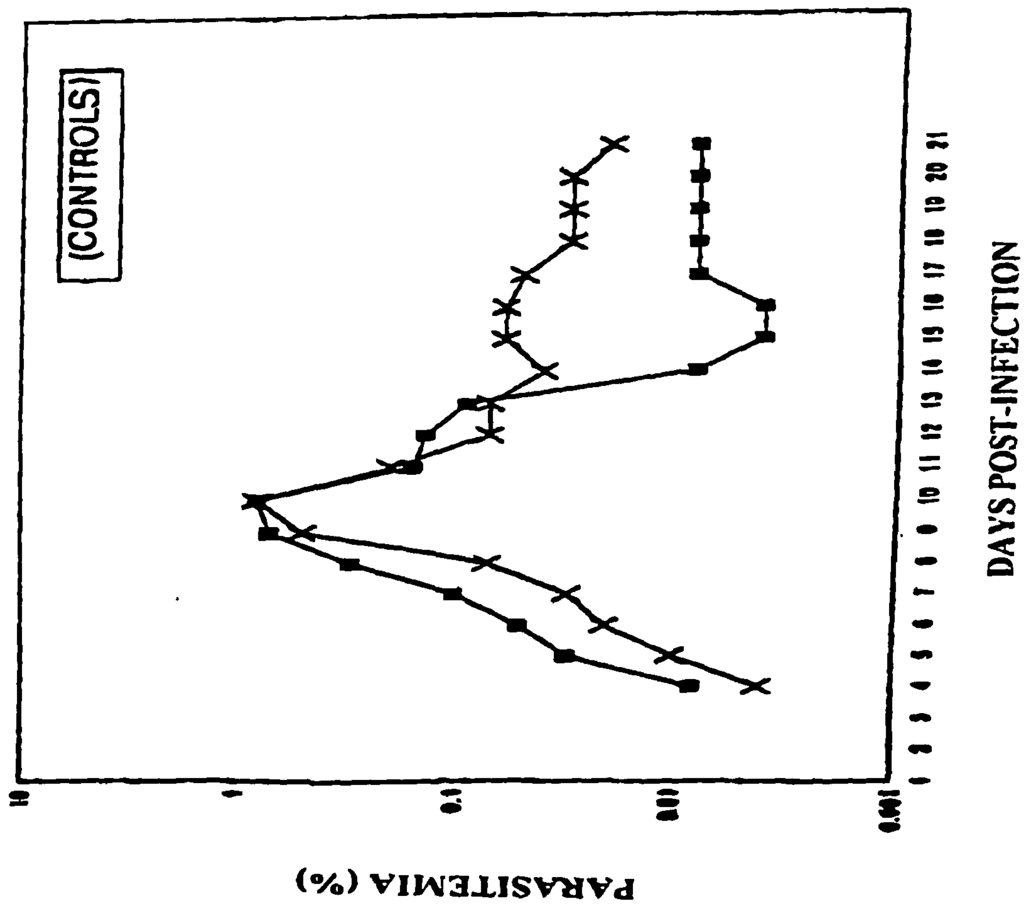


FIGURE 8C

FIGURE 8D

Vaccination test: recombinant MSP-1 (p19) of *Plasmodium cynomolgi* in the *Macaca sinica* toque macaque: second challenge infection

Year	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96	96
Month	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Days post-infection	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Number of days after challenge	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Vaccination p19																		
T 426	-	-	-	-	-	.008	-	-	-	-	-	-	-	-	-	-	-	-
T 427	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T 429	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Controls Physiological water RCA/FIA																		
T 436	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T 425	-	-	-	-	-	-	-	-	.008	-	-	-	-	-	-	-	-	-
T 438	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Controls Non vaccinated																		
T 448	.008	.03	.05	0.1	0.3	0.7	0.8	.16	.14	.09	.008	.004	.004	.008	.008	.008	.008	.008
T 449	.004	.01	.02	.03	0.07	0.5	0.8	0.2	.07	.07	.04	.06	.06	.05	.03	.03	.03	.02

- = Absence of parasites in 400 microscopic fields

FIGURE 9A

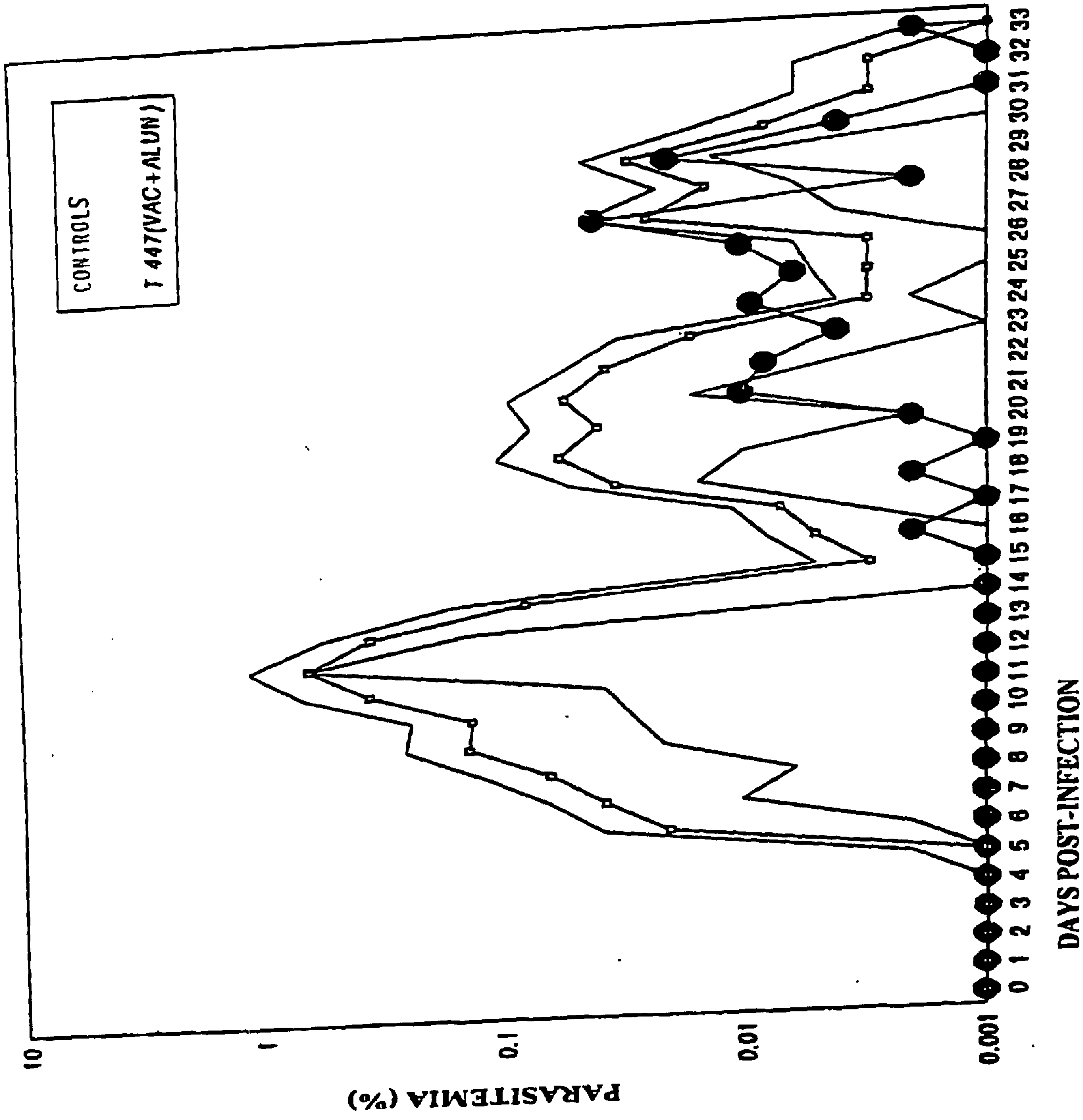
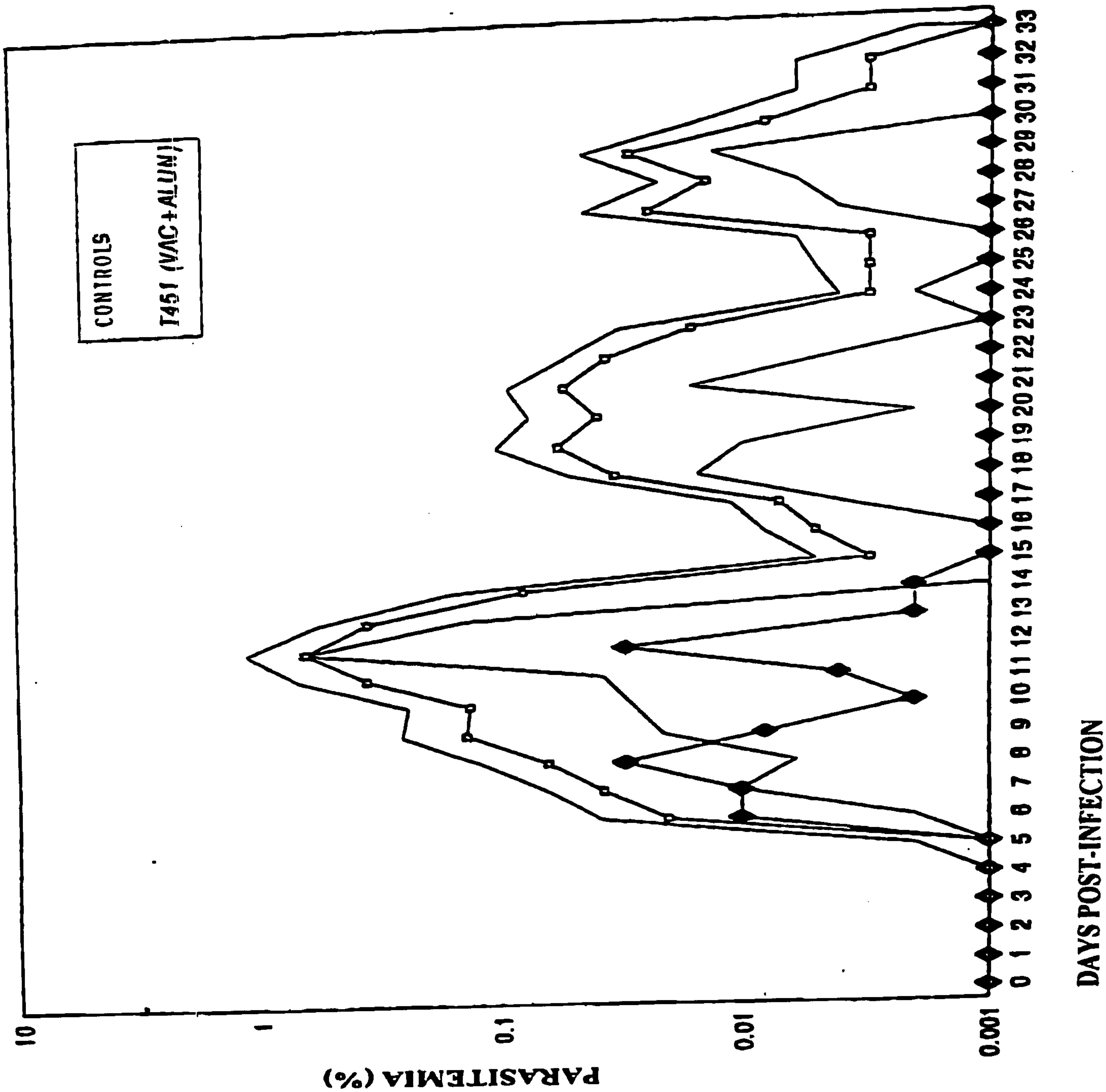

CONTROLS
T 447(VAC+ALUN)
PARASITEMIA (%)
10
1
0.1
0.01
0.001
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33
DAYS POST-INFECTION

FIGURE 9B



CONTROLS
T451 (VAC+ALUM)
PARASITEMIA (%)
10
1
0.1
0.01
0.001
DAYS POST-INFECTION
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33

FIGURE 9C

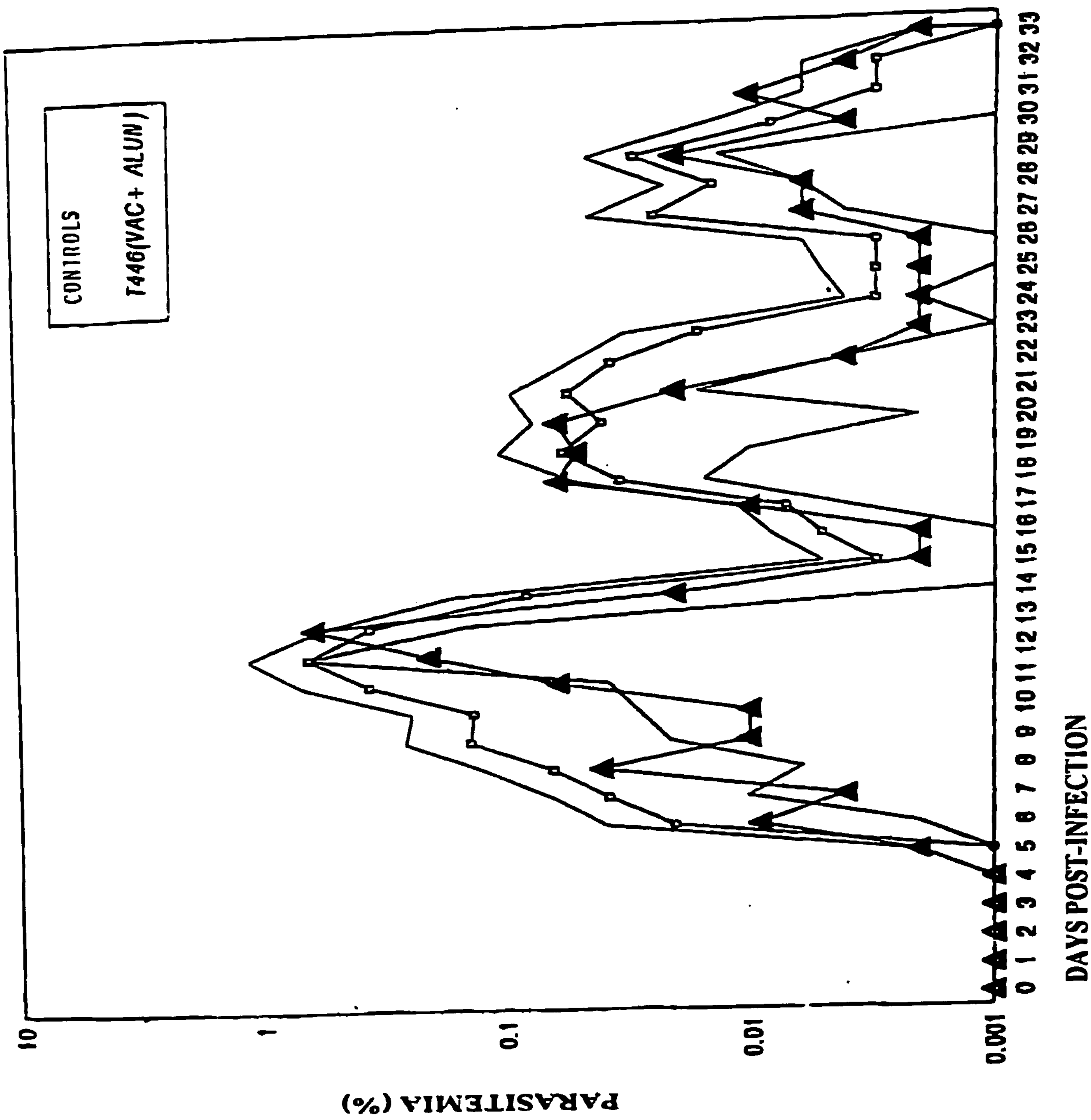
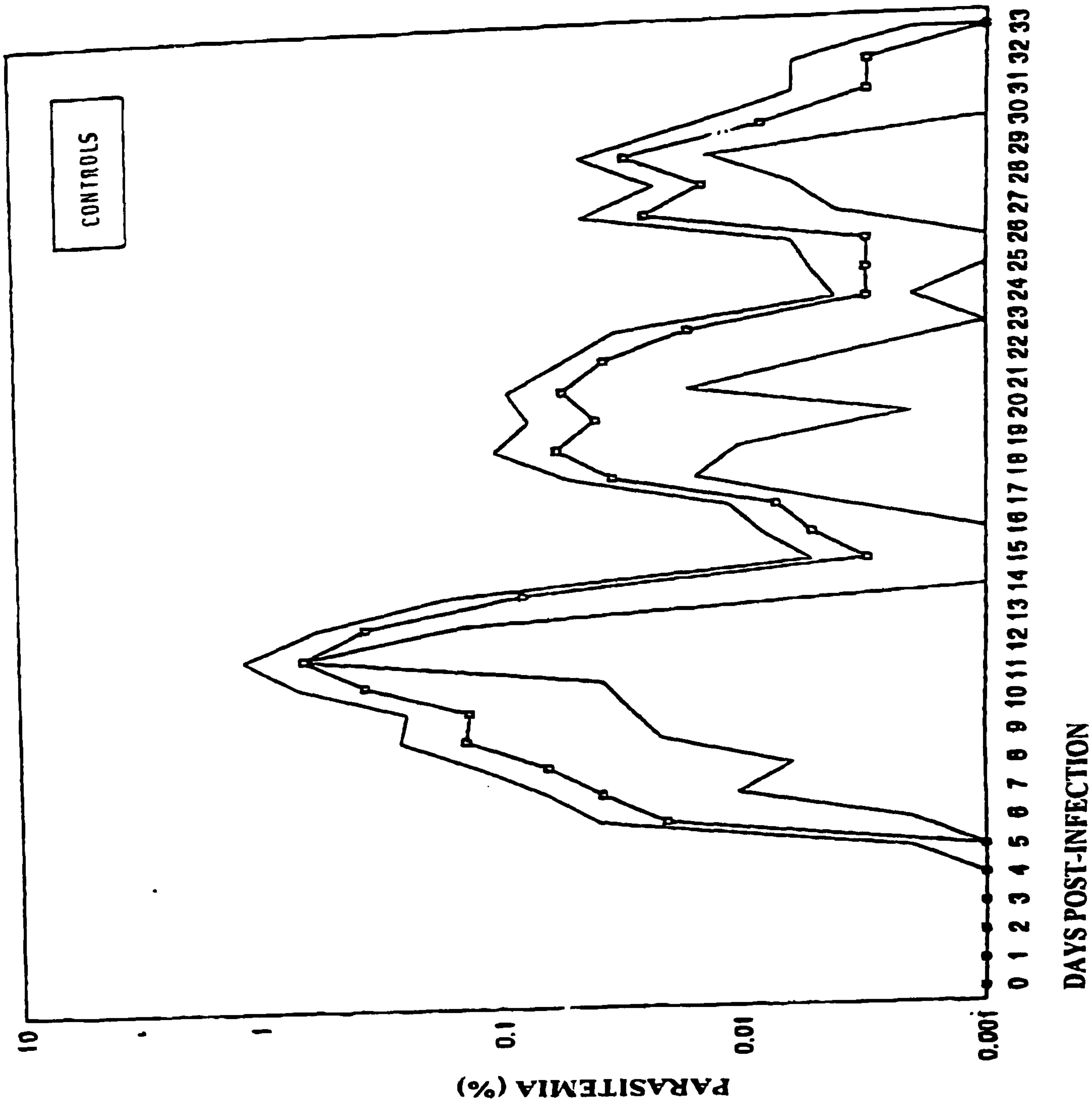

CONTROLS
T446(VAC+ ALUN)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33
DAYS POST-INFECTION
10
1
0.1
0.01
0.001
PARASITEMIA (%)

FIGURE 9D


CONTROLS
PARASITEMIA (%)
10
1
0.1
0.01
0.001
DAYS POST-INFECTION
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33

Vaccination test: *P. cynomolgi*/toque macaque with MSP-1 p19 of

P. cynomolgi in alum

Year	96	96	96	96	96	96	96	96	96	96	96	96	96
Month	7	7	7	7	7	7	7	7	7	7	7	7	7
Day	17	18	19	20	21	22	23	24	25	26	27	28	29
	5	6	7	8	9	10	11	12	13	14	15	16	17
GROUP 1													
(19 + ALUN)													
T 446	0.002	0.009	0.004	0.04	0.01	0.01	0.06	0.2	0.6	0.02	0.002	0.002	0.01
T 447	–	–	–	–	–	–	–	–	–	–	–	0.002	–
T 453	–	0.01	0.01	0.03	0.008	0.002	0.004	0.03	0.002	0.002	–	–	–
GROUP 2													
(NS + ALUN)													
T 450	0.002	0.01	0.05	0.04	0.06	0.04	0.12	0.2	0.12	0.02	0.006	0.01	0.01
T 454	0.002	0.05	0.06	0.14	0.3	0.28	0.8	1.3	0.63	0.02	0.002	0.002	0.01
T 455	–	–	–	0.008	0.05	0.08	0.14	0.4	0.3	0.2	0.002	0.002	0.002

– negative for parasites in 400 microscopic fields

FIGURE 9E(1)

Vaccination test: *P. cynomolgi*/toque macaque with MSP-1 p19 of

P. cynomolgi in alum

Year	96	96	96	96	96	96	96	95	96	96	96	96	96
Month	7	7	8	8	8	8	8	8	8	8	8	8	8
Day	30	31	1	2	3	4	5	6	7	8	9	10	11
	18	19	20	21	22	23	24	25	26	27	28	29	30
GROUP 1													
(19 + ALUN)													
T 446	0.06	0.05	0.06	0.02	0.004	0.002	0.002	0.002	0.002	0.006	0.006	0.02	0.004
T 447	0.002	–	0.002	0.01	0.008	0.004	0.009	0.006	0.01	0.04	0.002	0.02	0.004
T 453	–	–	–	–	–	–	–	–	–	–	–	–	–
GROUP 2													
(NS + ALUN)													
T 450	0.05	0.04	0.006	0.001	0.001	0.001	0.004	0.006	0.008	0.05	0.02	0.04	0.002
T 454	0.04	0.12	0.09	0.09	0.008	0.008	0.002	0.002	0.004	0.02	0.02	0.04	0.02
T 455	0.008	0.01	0.02	0.07	0.1	0.04	0.002	–	–	0.002	0.002	0.008	0.002

– negative for parasites in 400 microscopic fields

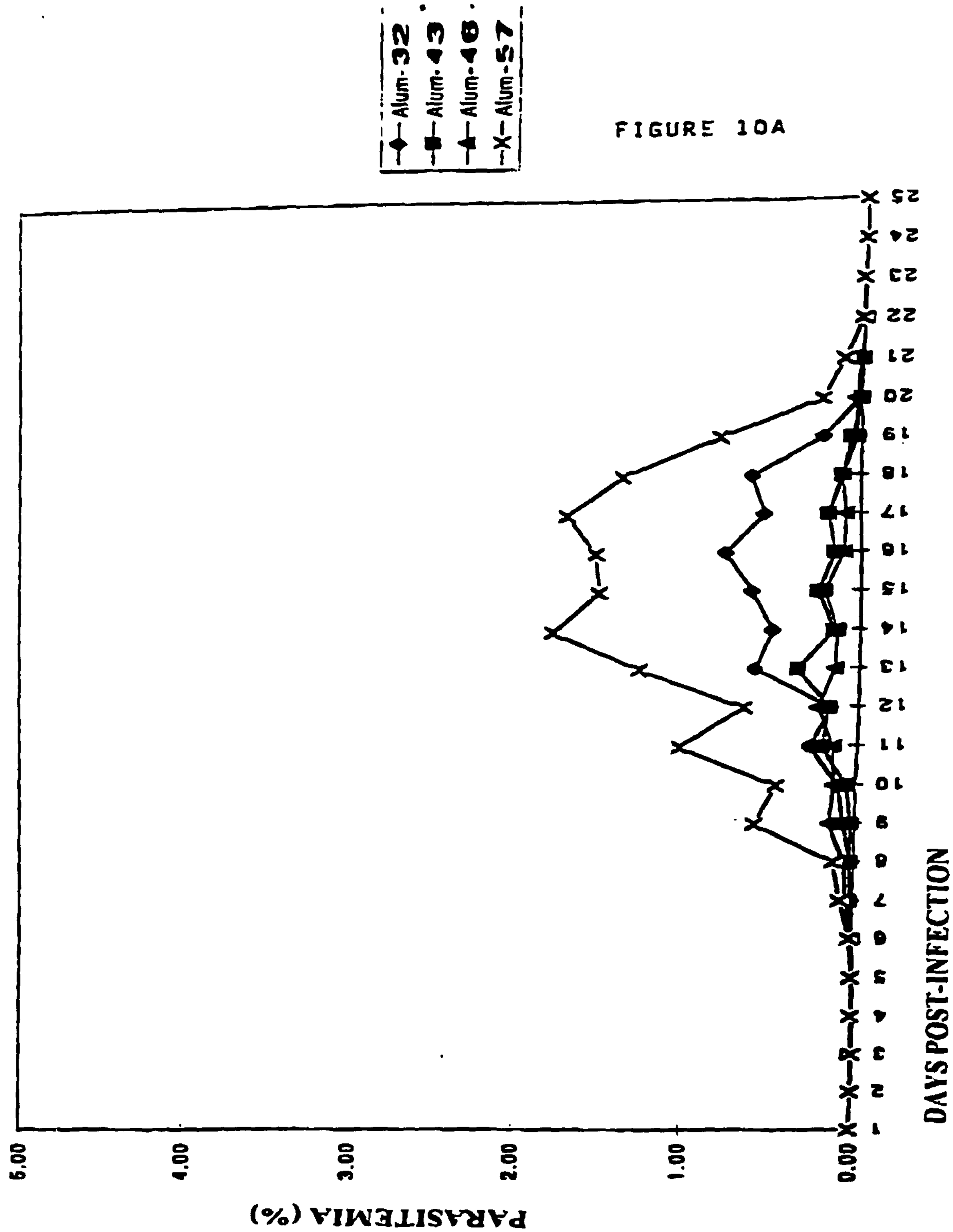
FIGURE 9E(2)

FIGURE 10A

PLASMODIUM FALCIPARUM VACCINATION TEST IN SAIMIRI

SCIUREUS SQUIRREL MONKEY

MSP-1 p19 VACCINATION WITH ALUM

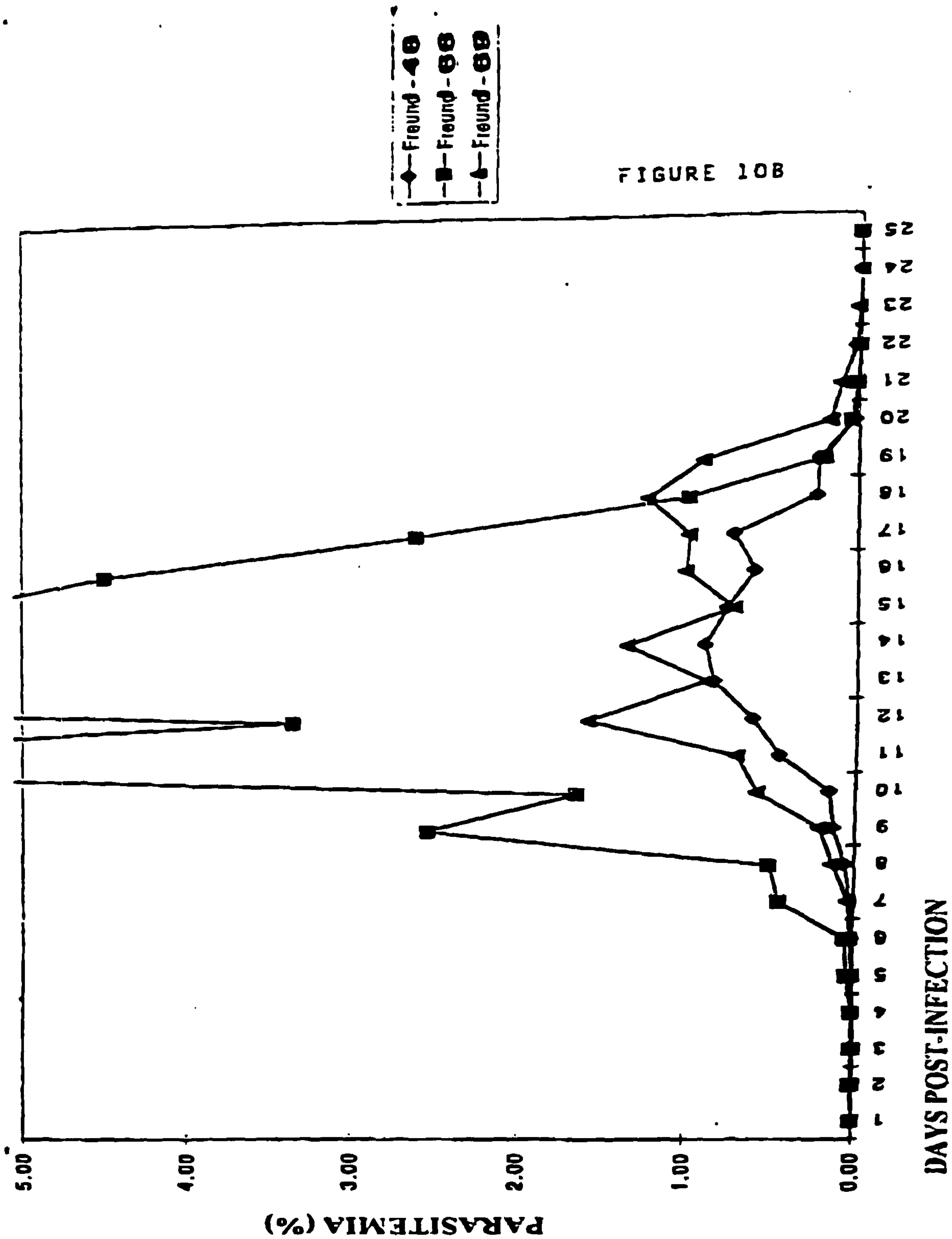


PLASMODIUM FALCIPARUM VACCINATION TEST IN SAIMIRI

SCIUREUS SQUIRREL MONKEY

MSP-1 p19 VACCINATION WITH FREUNDS

FIGURE 10B



PLASMODIUM FALCIPARUM VACCINATION TEST IN *SAIMIRI*

SCIUREUS SQUIRREL MONKEY

MSP-1 p19 VACCINATION WITH LIPOSOMES

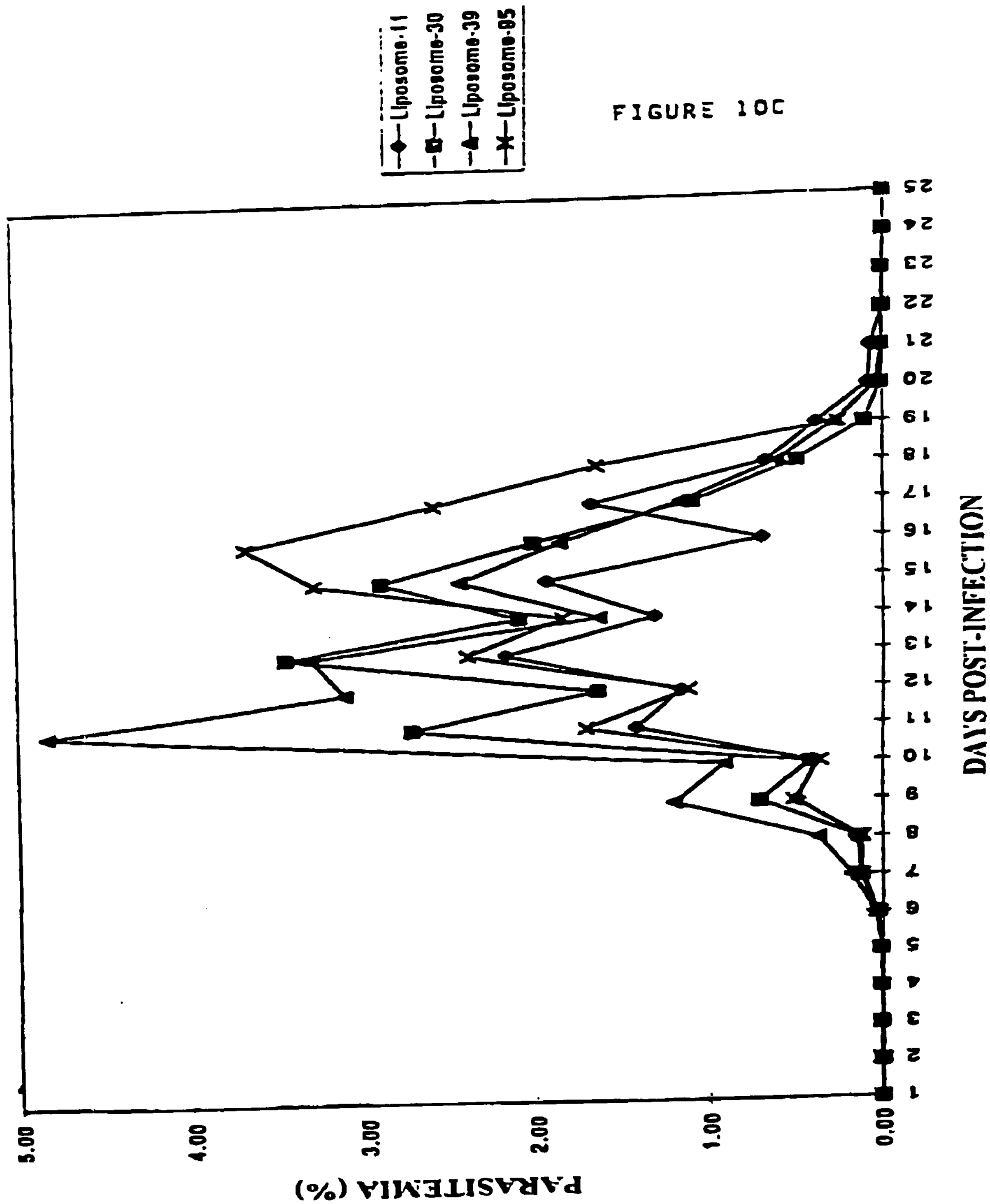


FIGURE 10C

PLASMODIUM FALCIPARUM VACCINATION TEST IN *SAIMIRI*

SCIUREUS SQUIRREL MONKEY

ALUM CONTROL

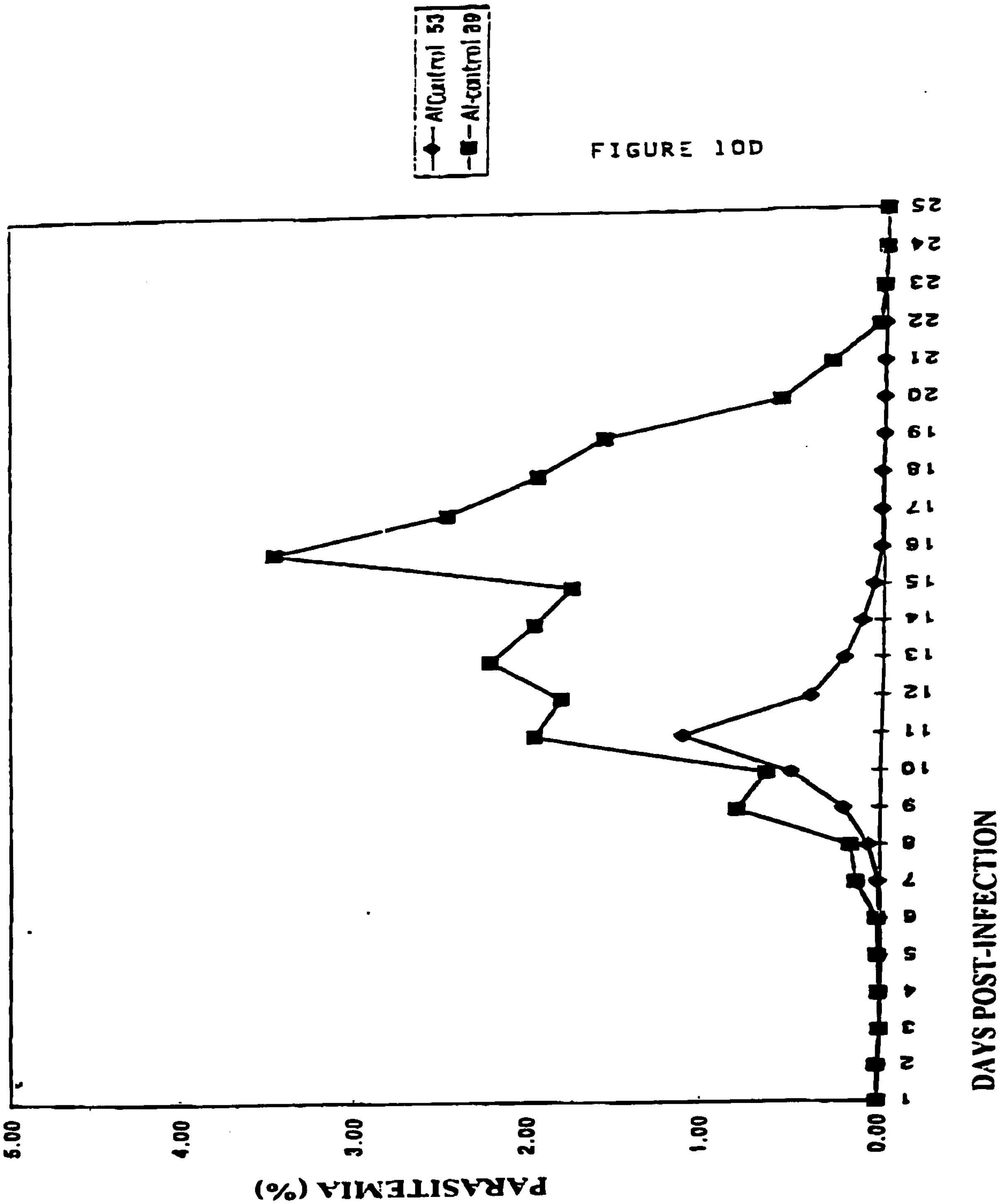


FIGURE 10D

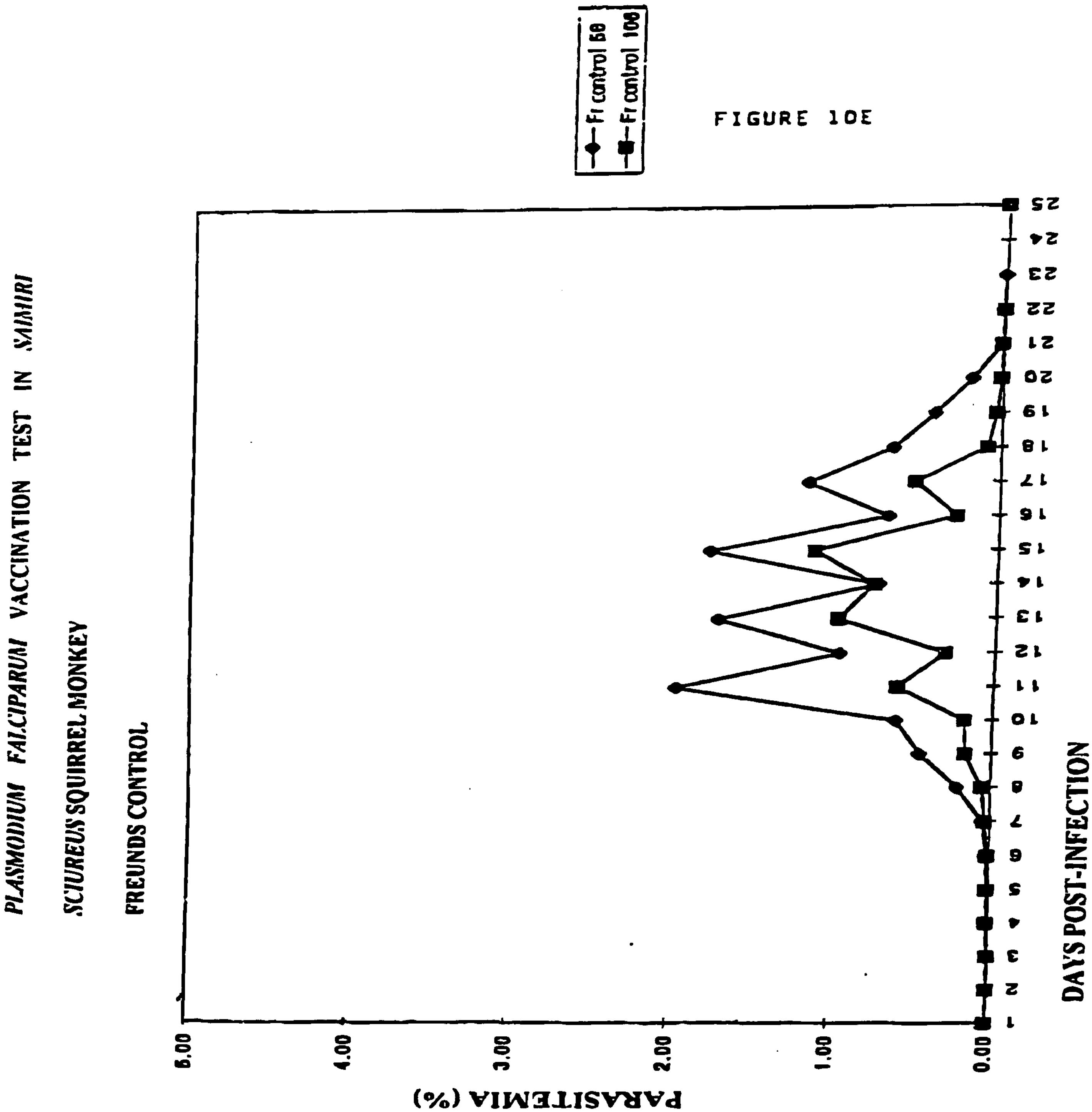

PLASMODIUM FALCIPARUM VACCINATION TEST IN SAIMIRI
SCIUREUS SQUIRREL MONKEY
FREUNDS CONTROL
Fr control 58
Fr control 106
PARASITEMIA (%)
0.00
1.00
2.00
3.00
4.00
5.00
DAYS POST-INFECTION
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

FIGURE 10E

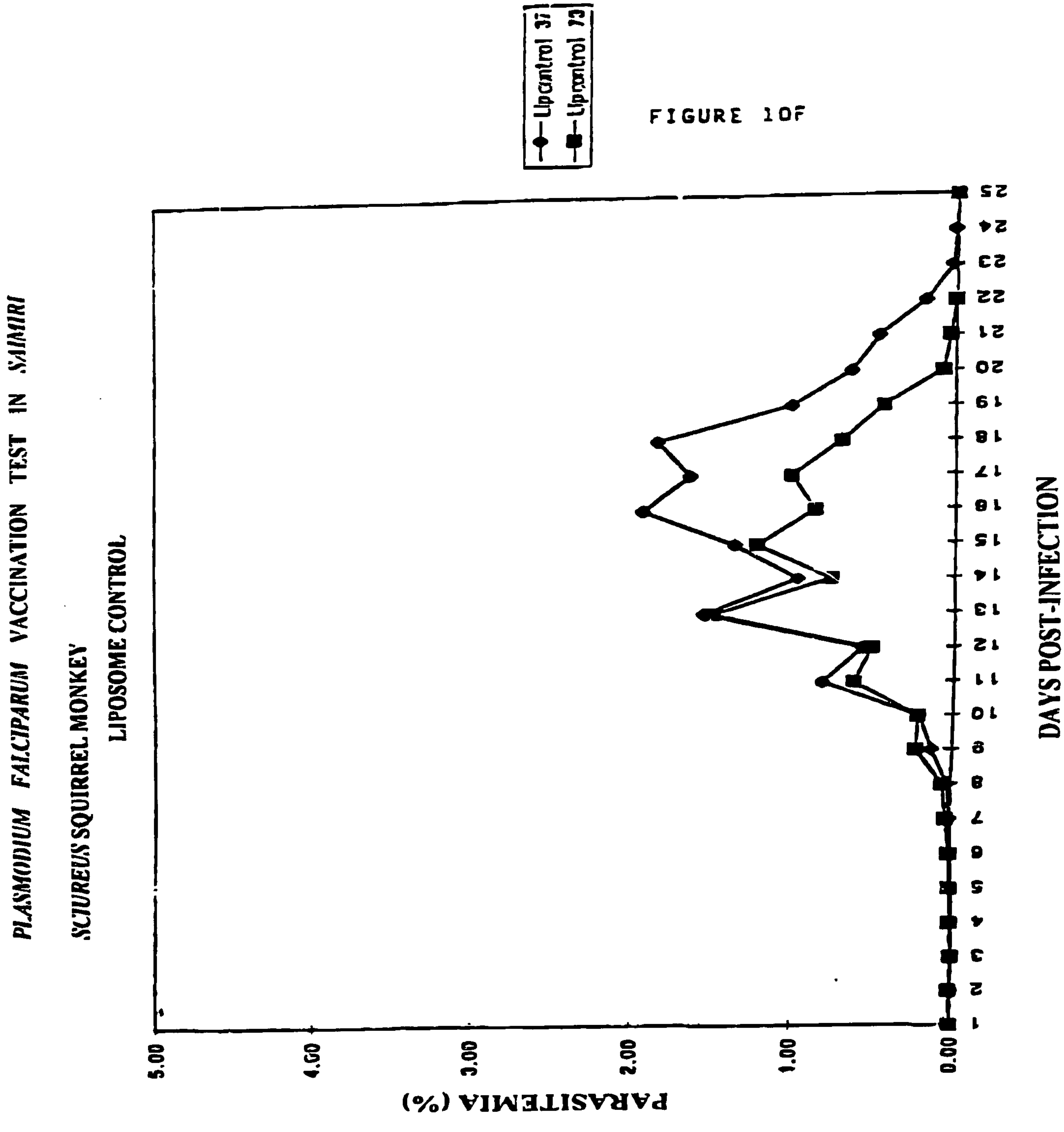

PLASMODIUM FALCIPARUM VACCINATION TEST IN SAIMIRI
SCIUREUS SQUIRREL MONKEY
LIPOSOME CONTROL
Lipcontrol 37
Lipcontrol 73
PARASITEMIA (%)
5.00
4.00
3.00
2.00
1.00
0.00
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
DAYS POST-INFECTION

FIGURE 10F

PLASMODIUM FALCIPARUM VACCINATION TEST IN *SAIMIRI*

SCIUREUS SQUIRREL MONKEY

PHYSIOLOGICAL WATER CONTROL

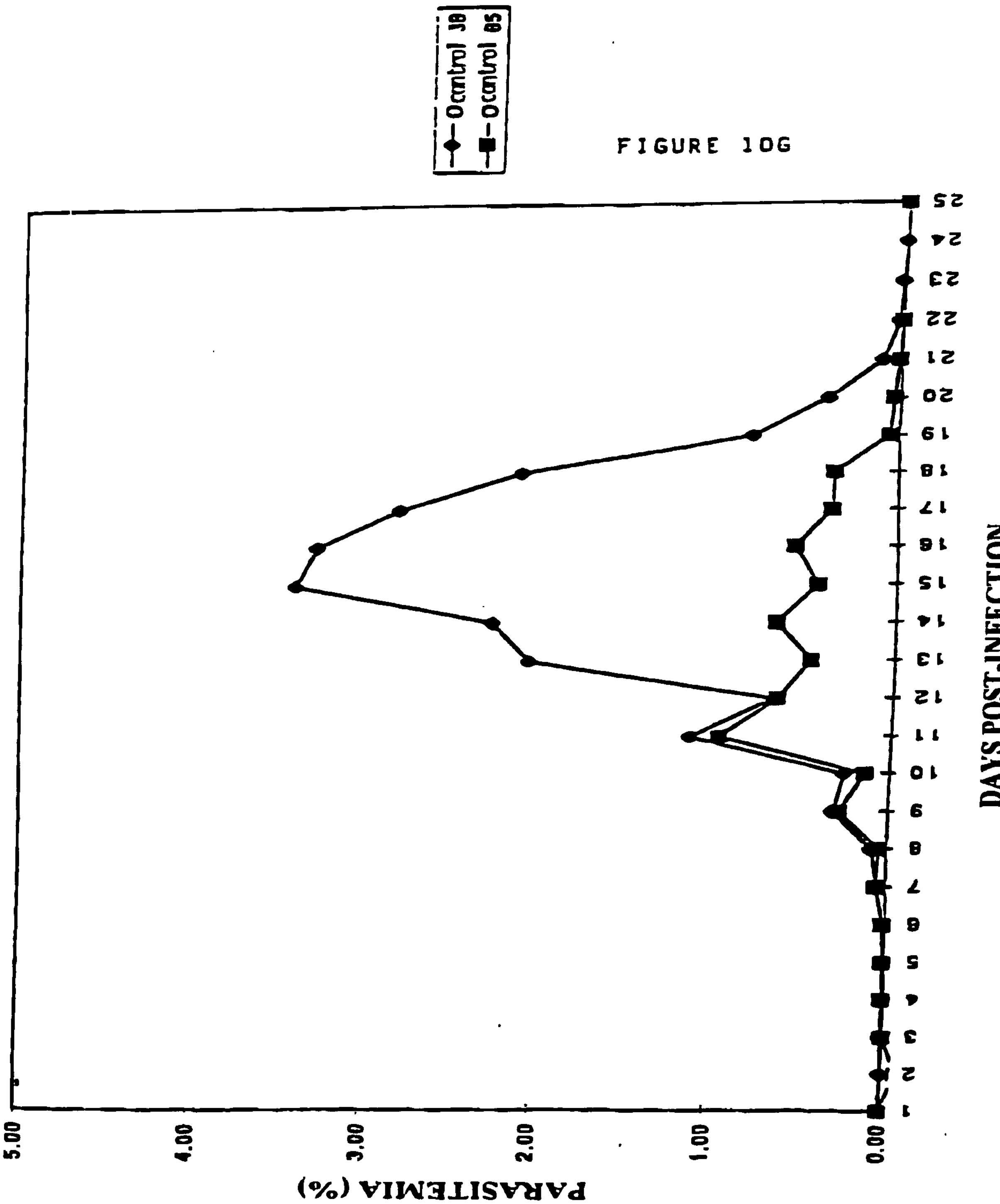


FIGURE 10G

PCT

ANNOUNCEMENT OF THE LATER PUBLICATION OF INTERNATIONAL SEARCH REPORTS

FILE

DEMANDE INTERNATIONALE ... EN MATIERE DE BREVETS (PCT)

(51) Classification internationale des brevets [6] : C12N 15/30, 15/86, C07K 14/445, 16/20

A3

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(74) Mandataires: GUTMANN, Ernest etc.; Ernest Gutmann-Yves Plasseraud S.A., 3, rue Chauveau-Lagarde, F-75008 Paris (FR).

(81) Etats désignés: AU, CA, CN, JP, KP, US, brevet européen (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Publiée

Avec rapport de recherche internationale.
Avant l'expiration du délai prévu pour la modification des revendications, sera republiée si de telles modifications sont reçues.

(88) Date de publication du rapport de recherche internationale: 31 décembre 1997 (31.12.97)

(54) Title: RECOMBINANT PROTEIN CONTAINING A C-TERMINAL FRAGMENT OF PLASMODIUM MSP-1

(54) Titre: PROTEINE RECOMBINANTE CONTENANT UN FRAGMENT C-TERMINAL DE MSP-1 TDE PLASMODIUM

(57) Abstract

The invention relates to a recombinant protein fabricated in a baculovirus system, of which the essential constitutive polypeptide sequence is that of a C-terminal fragment of 42 kilodaltons (p42) of the surface protein 1 (protein MSP-1) of the merozoite form of a parasite of the *Plasmodium* type, particularly *Plasmodium falciparum*, which is infectious for humans, said p42 fragment being particularly deleted of its region II and, if necessary, also of its region III. Said recombinant protein is applicable to the production of vaccines against malaria.

(57) Abrégé

L'invention concerne une protéine recombinante, fabriquée dans un système à baculovirus, dont la séquence polypeptidique constitutive essentielle est celle d'un fragment C-terminal de 42 kilodaltons (p42) de la protéine 1 de surface (protéine MSP-1) de la forme mérozoïte d'un parasite du type *Plasmodium*, en particulier *Plasmodium falciparum*, infectieux pour l'Homme, ce fragment p42 étant particulièrement délété de sa région II et, le cas échéant, aussi de sa région III. Cette protéine recombinante est applicable à la production de vaccins contre la malaria.

INTERNATIONAL SEARCH REPORT

Intern   nal Application No
PCT/FR 97/00291

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/30 C12N15/86 C07K14/445 C07K16/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MOLECULAR AND BIOCHEMICAL PARASITOLOGY, vol. 74, no. 1, 20 December 1995, pages 105-111, XP000603955 LONGACRE, S.: "The Plasmodium cynomolgi merozoite surface protein 1 C-terminal sequence and its homologies with other Plasmodium species" cited in the application see page 107, column 1, line 4 - page 109, column 1, line 17 see page 109, column 1, line 5 - line 11; figures 1,2	1

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 August 1997	26-11-1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	CHAMBONNET, F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/FR 97/00291

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	MOLECULAR AND BIOCHEMICAL PARASITOLOGY, vol. 64, no. 2, 1 January 1994, pages 191-205, XP000603954 LONGACRE, S. ET AL.: "Plasmodium vivax merozoite surface protein 1 C-terminal recombinant proteins in baculovirus" cited in the application see the whole document ---	20
A	EP 0 154 454 A (WELLCOME FOUND) 11 September 1985 see claims -----	1

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No
PCT/FR 97/00291

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons

1. ☐ Claims Nos
because they relate to subject matter not required to be searched by this Authority, namely

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows

1. Claims 1-14, 21, - and in part 20, 22, 23
2. Claims 15-19, 34, 35,
3. Claims 20, 22-23 in pa

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims: it is covered by claims Nos :

1-14, 21, 36-38 and in part 20, 22, 23

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

...formation on patent family members

International Application No
PCT/FR 97/00291

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0154454 A	11-09-85	AU 592749 B	25-01-90
		AU 3904685 A	05-09-85
		DK 79985 A	23-08-85
		GB 2154592 A	11-09-85
		IL 74409 A	26-08-94
		JP 2584733 B	26-02-97
		JP 61019490 A	28-01-86
		JP 6189772 A	12-07-94
		PH 25993 A	13-01-92
		US 5597708 A	28-01-97

RAPPORT DE RECHERCHE INTERNATIONALE

Demande internationale No

PCT/FR 97/00291

A. CLASSEMENT DE L'OBJET DE LA DEMANDE
CIB 6 C12N15/30 C12N15/86 C07K14/445 C07K16/20

Selon la classification internationale des brevets (CIB) ou à la fois selon la classification nationale et la CIB

B. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE

Documentation minimale consultée (système de classification suivi des symboles de classement)
CIB 6 C07K

Documentation consultée autre que la documentation minimale dans la mesure où ces documents relèvent des domaines sur lesquels a porté la recherche

Base de données électronique consultée au cours de la recherche internationale (nom de la base de données, et si cela est réalisable, termes de recherche utilisés)

C. DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie *	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
A	MOLECULAR AND BIOCHEMICAL PARASITOLOGY, vol. 74, no. 1, 20 Décembre 1995, pages 105-111, XP000603955 LONGACRE, S.: "The Plasmodium cynomolgi merozoite surface protein 1 C-terminal sequence and its homologies with other Plasmodium species" cité dans la demande voir page 107, colonne 1, ligne 4 - page 109, colonne 1, ligne 17 voir page 109, colonne 1, ligne 5 - ligne 11; figures 1,2 ---	1

-/--

[X] Voir la suite du cadre C pour la fin de la liste des documents

[X] Les documents de familles de brevets sont indiqués en annexe

* Catégories spéciales de documents cités:

"A" document définissant l'état général de la technique, non considéré comme particulièrement pertinent

"E" document antérieur, mais publié à la date de dépôt international ou après cette date

"L" document pouvant jeter un doute sur une revendication de priorité ou cité pour déterminer la date de publication d'une autre citation ou pour une raison spéciale (telle qu'indiquée)

"O" document se référant à une divulgation orale, à un usage, à une exposition ou tous autres moyens

"P" document publié avant la date de dépôt international, mais postérieurement à la date de priorité revendiquée

"T" document ultérieur publié après la date de dépôt international ou la date de priorité et n'appartenenant pas à l'état de la technique pertinent, mais cité pour comprendre le principe ou la théorie constituant la base de l'invention

"X" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme nouvelle ou comme impliquant une activité inventive par rapport au document considéré isolément

"Y" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme impliquant une activité inventive lorsque le document est associé à un ou plusieurs autres documents de même nature, cette combinaison étant évidente pour une personne du métier

"&" document qui fait partie de la même famille de brevets

1

Date à laquelle la recherche internationale a été effectivement achevée	Date d'expédition du présent rapport de recherche internationale
21 Août 1997	26 -11- 1997
Nom et adresse postale de l'administration chargée de la recherche internationale Office Européen des Brevets, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fonctionnaire autorisé CHAMBONNET, F

Formulaire PCT/ISA/210 (deuxième feuille) (juillet 1992)

RAPPORT DE RECHERCHE INTERNATIONALE

Demande internationale No

PCT/FR 97/00291

C.(suite) DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie *	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
A	MOLECULAR AND BIOCHEMICAL PARASITOLOGY, vol. 64, no. 2, 1 Janvier 1994, pages 191-205, XP000603954 LONGACRE, S. ET AL.: "Plasmodium vivax merozoite surface protein 1 C-terminal recombinant proteins in baculovirus" cité dans la demande voir le document en entier ---	20
A	EP 0 154 454 A (WELLCOME FOUND) 11 Septembre 1985 voir revendications -----	1,10

RAPPORT DE RECHERCHE INTERNATIONALE

Demande internationale n°
PCT/FR 97/00291

Cadre I Observations - lorsqu'il a été estimé que certaines revendications ne pouvaient pas faire l'objet d'une recherche (suite du point 1 de la première feuille)

Conformément à l'article 17.2)a), certaines revendications n'ont pas fait l'objet d'une recherche pour les motifs suivants.

1. ☐ Les revendications n^{os}
se rapportent à un objet à l'égard duquel l'administration n'est pas tenue de procéder à la recherche, à savoir:

2. ☐ Les revendications n^{os}
se rapportent à des parties de la demande internationale qui ne remplissent pas suffisamment les conditions prescrites pour qu'une recherche significative puisse être effectuée, en particulier:

3. ☐ Les revendications n^{os}
sont des revendications dépendantes et ne sont pas rédigées conformément aux dispositions de la deuxième et de la troisième phrases de la règle 6.4.a).

Cadre II Observations - lorsqu'il y a absence d'unité de l'invention (suite du point 2 de la première feuille)

L'administration chargée de la recherche internationale a trouvé plusieurs inventions dans la demande internationale, à savoir:

1. REVENDICATIONS 1-14, 21, 36-38 et partiellement 20, 22, 23
2. REVENDICATIONS 15-19, 34, 35, 39
3. REVENDICATIONS 20, 22-23 partiellement

1. ☐ Comme toutes les taxes additionnelles ont été payées dans les délais par le déposant, le présent rapport de recherche internationale porte sur toutes les revendications pouvant faire l'objet d'une recherche.

2. ☐ Comme toutes les recherches portant sur les revendications qui s'y prêtaient ont pu être effectuées sans effort particulier justifiant une taxe additionnelle, l'administration n'a sollicité le paiement d'aucune taxe de cette nature.

3. ☐ Comme une partie seulement des taxes additionnelles demandées a été payée dans les délais par le déposant, le présent rapport de recherche internationale ne porte que sur les revendications pour lesquelles les taxes ont été payées, à savoir les revendications n^{os}

4. ☒ Aucune taxe additionnelle demandée n'a été payée dans les délais par le déposant. En conséquence, le présent rapport de recherche internationale ne porte que sur l'invention mentionnée en premier lieu dans les revendications; elle est couverte par les revendications n^{os}
1-14, 21, 36-38 et partiellement 20, 22, 23

Remarque quant à la réserve

☐ Les taxes additionnelles étaient accompagnées d'une réserve de la part du déposant.

☐ Le paiement des taxes additionnelles n'était assorti d'aucune réserve.

RAPPORT DE RECHERCHE INTERNATIONALE

Renseignements relatifs aux membres de familles de brevets

Demande internationale No
PCT/FR 97/00291

Document brevet cité au rapport de recherche	Date de publication	Membre(s) de la famille de brevet(s)	Date de publication
EP 0154454 A	11-09-85	AU 592749 B	25-01-90
		AU 3904685 A	05-09-85
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